DEVELOPMENT OF A RECOMBINANT PROTEIN FOR THE IDENTIFICATION OF *SARCOCYSTIS NEURONA* INFECTIONS IN HORSES

By

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DEDICTED TO VAUNTED VAMP

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ABSTRACT

Equine protozoal myeloencephalitis is an infectious parasitic infection of horses caused by *Sarcocystis neurona* that results in devastating neurologic disease. Factors surrounding the pathogenesis of infection in the horse are unclear, but it is currently believed the horse is an aberrant host. The diagnosis of equine protozoal myeloencephalitis has been based on serum and cerebral spinal fluid antibodies to *Sarcocystis neurona* derived antigens demonstrated by immunoblot. The antigens used in the diagnosis of equine protozoal myeloencephalitis have not been demonstrated to be species specific or diagnostic.

Sarcocystis neurona merozoites were grown in cultured cells and a novel method for parasite release was developed. Parasite derived antigens were separated by electrophoresis and used in immunoassays to identify antigens recognized by antibodies from infected horses, but not recognized by those from uninfected horses. A major surface antigen was characterized. The nucleotide sequence of a gene encoding a major surface antigen of *S. neurona* was cloned and sequenced. Oligonucleotides were developed to amplify an RT-PCR product from *Sarcocystis neurona* RNA.

A cDNA library was prepared from the mRNA of *S. neurona* and cloned into an expression vector. A cDNA clone encoding a putative surface antigen was identified among 96 clones characterized by preparing sequence tags. The sequence tag encoded a peptide with significant sequence similarity to a segment of the major surface antigen of *S. muris*. The cDNA library was screened with the partial nucleotide sequence as a probe, and a clone containing the sequence of the full coding region of SnMSA-1 was obtained. Southern and Northern analysis of the SnMSA-1 gene revealed a single copy in the genomic DNA of *S. neurona* cultured merozoites and an abundance of mRNA in the merozoite. A recombinant form was produced, rSnMSA-1, and purified using an affinity column. Monoclonal antibodies raised against merozoites recognized the recombinant protein overexpressed in pET 14b. Studies of the native and recombinant protein confirmed that the antigen was a 29 kDa protein with a pI of 7.3 located on the surface of the parasite. Immunoblotted rSnMSA-1 confirmed the presence of antibodies to this antigen in serum and cerebrospinal fluids from horses with clinical EPM. Antibodies raised against rSNMSA-1 were useful for the quantitation of antigen in preparative samples and in the identification of parasites in clinical tissues.

CHAPTER 1
INTRODUCTION

Equine Protozoal Myeloencephalitis

Equine protozoal myeloencephalitis (EPM) is a parasitic infection of horses caused by *Sarcocystis neurona* that results in devastating neurologic disease. All sectors of the equine community are influenced with what is perceived as an epidemic of EPM. The apprehension of the horse industry is due to serological data that estimate the prevalence of infection in the equine population is at least fifty percent.[1] It is unclear that fifty per cent of the animals have been exposed to *S. neurona*. Low test specificity with the commercial immunoblot fosters anxiety and misunderstanding of this disease. The first rational step to the development of a sero-diagnostic test is the identification and characterization of species-specific antigens that induce an immune response in the horse. Current molecular techniques make the identification and production of recombinant protein antigens possible. These recombinant antigens will form the basis of a specific test for EPM.

Sarcocystis

Coccidia are obligatory intracellular apicomplexan protozoa whose life cycles notably consist of merogony, gametogony, and sporogony.[2] The life cycle is completed in either one or two hosts. If in two hosts, the asexual phase is completed in the intermediate

host and the sexual phase in the definitive host. The taxonomic criteria used in identifying coccidia to genus have been cyst and oocyst morphology and life cycle. Uncertainty with the traditional criteria used to distinguish species arises because some morphological features are subject to change during the development of the parasite and the complete life cycles of many organisms currently classified as coccidia are still unknown. The recent development of molecular methods to recognize various species of coccidia provides powerful tools to reevaluate the accepted taxonomy. Few molecular data are available for the named 189 species however, and little is known at present about the genetic divergence among species of *Sarcocystis*. In the natural hosts, sarcocystosis is usually benign causing little pathology in either host. However, disease has been recognized with several *Sarcocystis* species, generally involving merogony of the parasite in the intermediate host.

Sarcocystis Neurona Infection in Horses

Equine sarcocystosis caused by *S. neurona* causes a neurologic disease of horses in the Americas, found only in the Western hemisphere.[3] The most typical presentation is a lame horse, but animals may alternatively present with primary brain disease or a combination. The disease has no defining signs because the parasite can inhabit any area of the central nervous system (CNS) of the horse. Clinically localizing CNS signs are a direct reflection of the site of pathology and are quite specific. *Sarcocystis neurona* infections can range from subtle to severe involving only the brain, spinal cord, or both. The disease is usually progressive. A prototypical lesion is a mixed inflammatory cell response with neuronal destruction and protozoa may or may not be found in the lesion.[4]

The typical spinal cord lesions were reported in horses in Kentucky by Rooney and co-workers in 1964 and recognized in the CNS of Brazilian horses by Macruz et al. at about the same time.[5-6] Protozoa were first identified in EPM lesions in 1974.[6-8] The etiology was originally thought to be *Toxoplasma gondii*; however, with improved diagnostics the parasite was recognized as a *Sarcocystis* species.[8-12] The organism that causes EPM in horses was named *Sarcocystis neurona* in 1991 based on structural similarity to schizonts of *Sarcocystis* and *Frenkelia*.[6]

Sarcocystis neurona

Dubey et al. noted that merozoites of *S. neurona* lack rhoptries, lack a parasitophorous vacuole in the tissues examined, and divide by endopolygeny.[6] The absence of rhoptries in merozoites distinguishes *S. neurona*, other species of *Sarcocystis*, and *Frenkelia* from any other cyst-forming coccidian parasite found in domestic animals. In addition to structural data, Dubey et al. reported antigenic differences among *S. neurona* and *T. gondii, Neospora caninum, Hammondia hammondi,* and *Caryospora bigenetica.*[6] Dubey et al. reported positive cross-reactivity of *S. neurona* merozoites with *S. cruzi* antiserum interpreting these data as indicative of a close relationship with other species of *Sarcocystis*.[6] The cross-reactive nature of antigens of *Sarcocystis* has been reported and can not serve as a tool to differentiate species within the genus.[12] *Sarcocystis neurona* is closely related to *S. falcatula* and *S. muris* based on rRNA sequence analysis and shares cross-reactive antigens with other *Sarocystis sp.*[7] The historical criterion for differentiation of species of *Sarcocystis* has been the structure of the sarcocyst wall in the intermediate host. In the case of *S. neurona,* no cysts have been identified and the

intermediate host is unknown. That notwithstanding, Dubey and colleagues used the structure of the asexual stage, merozoites and schizonts, and its putative predilection to localize in the CNS of the horse as criteria to name this "new" species. Additionally, they described the presence of a residual body in association with *S. neurona* schizonts that is absent in other *Sarcocystis* species studied so far. Lastly, the researchers were unable to induce encephalomyelitis by inoculating horses with the oocysts of ten other species of *Sarcocystis.*[12]

When Dubey et al. conducted the animal trials, they used traditional methods to define the *Sarcocystis spp.* that were administered to horses. Studies done by Box and colleagues elucidating the life cycle of *Sarcocystis falcatula* that uses the opossum (*Didelphis virginiana*) and the brown-headed cowbird (*Molothrus ater*) as hosts indicate that the definitive host may harbor more than one *Sarcocystis* species.[13-16] Also interesting was the observation in Box's work that the parasite could develop to different degrees in different intermediate hosts. Entzeroth and co-workers noted varied associations between the parasite-host cell relationships during the maturation of stages of infection in different organs.[17] They concluded further studies were needed to determine if the variations observed were due to the species characteristics of *Sarcocystis* or were influenced by the type of host cell involved.[17] Recent evidence indicates that infected opossums shed sporocysts of *Sarcocystis neurona* as well as three other *Sarcocystis spp.*[18-19] Despite this advance, the complete parasite life-cycle has not been demonstrated for *Sarcocystis neurona* nor have Koch's postulates been unequivocally fulfilled for transmitting disease in the horse, underscoring the complexity of the host-parasite relationship.

Equine Response to Sarcocystosis

Toxoplasma *gondii* is an apicomplexan parasite that has been shown to infect horses when fed oocysts resulting in serological conversion and either persistent infection or resolution of infection.[20] The same study showed that in some cases antibody titer was negative by complement-dependent serum neutralization and for agglutinating IgG against whole *T. gondii*, but animals were shown to harbor viable *T. gondii*. Another apicomplexan, *Neospora*, has been isolated from the CNS of horses and suspected as a cause of equine abortion.[21-23] *Neospora* can be confounding in EPM diagnosis if it is not considered. The source of the error is in missing the diagnosis of EPM if no *S. neurona* antibodies are present. Alternatively, an error that attributes the EPM state to *S. neurona* because *S. neurona* antibodies are present even though *N. hughesi* is the culprit.[22] To rule out *Neospora* as the etiologic agent, one must screen by immunoblot using *Neospora* antigens or confirm the diagnosis by isolating and identifying the infecting organism.[22]

Several *Sarcocystis* species can produce muscle cysts in the horse. These include *S. bertrami*, *S. equicanis* and *S. fayeri* and all use the dog as a definitive host.[1] These species are differentiated by cyst wall morphology. Unfortunately, the cyst wall changes with maturation resulting in confusion in the identification of cysts.[24] The schizont was reported as the immune stimulus in *S. fayeri* sarcocystosis in the horse.[25] An interesting suggestion is that horses suffering severe muscle sarcocystosis exhibit weakness and disease due to a toxin.[26] Saito et al. demonstrated the extract of *Sarcocystis cruzi* cysts from bovine muscle contained a crude toxin that was a water soluble, acid-alkali stable and thermolabile protein with estimated molecular mass of 15-16 kDa.[26]

When sporocysts of *S. neurona* were identified in an infected opossum, the brown-headed cowbird was speculated to be the intermediate host and *S. falcatula* was considered similar, if not the same, organism.[27] Feeding trials as well as biologic studies were to show *S. neurona* and *S. falcatula* were indeed very close though distinct species.[28-29] Cutler et al. showed that seroconversion to *S. neurona* did not occur when *S. falcatula* sporocysts were given orally to horses.[28] When Fenger et al. fed up to twenty million sporocysts from feral opossums to each of five foals they did observe seroconversion as well as demonstrable antibodies in the CSF to *S. neurona*; however, no gross lesions were seen at the end of the trial.[30] Clinical signs were reported in all foals in Fenger's trial but protozoa were not observed in tissue sections and no protozoa were cultured from spinal cord samples. The inoculating sporocysts were unidentified in this study except originating from feral opossums. Further, *Neospora* was not considered as a control.

The pathogenesis of sarcocystosis in several species has been documented.[2,31-34] Intermediate hosts acquire infection by ingesting sporocysts that are shed in the feces of infected definitive hosts. Definitive hosts become infected by ingesting the encysted form (sarcocysts) of the parasite in the muscle of the intermediate host. Horses are thought to ingest sporocysts shed from the opossum in contaminated feed, water, or pasture. After sporozoites are released from the sporocysts in the intestine of the horse, they are assumed to undergo at least one merogonous generation. In other *Sarcocystis* spp., schizogony occurs in the endothelial cells of blood vessels of the host and this may occur in the horse; what remains to be demonstrated is the isolation of merozoites from the blood of an infected horse. It is possible that two rounds of schizogony occur in mononuclear cells or

the vascular endothelium (arteriolar followed by capillary endothelium). Immune responses, including antibody production, are induced as merozoites pass through the vascular endothelium of the blood-brain barrier to the immune privileged central nervous system, where they survive.[35-37]

Hematogenous dissemination of the parasite would result in many tissues containing the pre-cyst form of the protozoa. The parasite is an obligate intracellular organism and one would expect cellular immunity to play a large role in the host's response to infection. In some intermediate hosts *Sarcocystis* spp. have been demonstrated in the blood and transmitted by blood transfusion.[35-41] Because the parasite surface antigens are exposed to the host immune system during hematogenous dissemination of the parasite, one can expect an antibody response by the host. When *Sarcocystis* spp. that can complete the life cycle in the horse infect horse muscle, one would expect the hematogenous phase to have induced an immune response. Protective antibodies against infection with *S. cruzi* have been induced in calves in studies using *S. cruzi* sporocysts to challenge calves after initial exposure and development of an immune response. These studies confirm the presence of an immune system response.[42] *S. fayeri* uses the horse as an intermediate host; however, there was no correlation between *S. fayeri* infection and EPM when the immune response of the EPM infected horse is examined by immunoblot using *S. neurona* antigens derived from cultured merozoites.[12]

Parasite Antigens

The comparison of the immune response in EPM infected horses to that in other equine sarcocystis infections should be performed on corresponding life cycle stages to produce meaningful data. Any other comparison would be misleading. Diagnosis of *S. neurona* infection by western blot analysis is based on the observation that *S. neurona* specific antibodies are present in equine serum and CSF samples from affected horses.[43-45] A Western blot for *S. neurona* antibody has subsequently been made commercially available.[43] The low specificity and high sensitivity of immunoblot in the absence of clinical signs predicts a high incidence of infection.[11] Unpublished data indicated that preliminary morphologic, immunologic, and DNA comparisons have detected only minor differences among isolates, suggesting that the diagnostic antigens were shared among isolates. Should *S. neurona* differ antigenically among various isolates this could have profound impact on the interpretation of test results. Accommodating any antigenic differences among isolates is critical to standardizing the diagnostic test. Cross-reactive antigens among *Sarcocystis* species may be one of several possibilities for the low specificity of current commercial immunoblot tests to detect infected horses. Basic questions remain including the following: which antigens are relied upon in current tests and are they species specific? The identification of a species specific antigen is critical to the diagnosis of clinical cases of EPM.

Diagnostic Antigens in the horse

Reports identifying and verifying the species specificity of the diagnostic antigens of *S. neurona* are limited however, histopathologically verified EPM horses consistently

detected protein bands at 22.5, 13 and 10.5 kDa on a Western blot.[12] These bands were not detected by rabbit immune sera to *S. muris*, *S. cruzi* and sera from a pony experimentally infected with *S. fayeri*.[12] Dubey and others have based serologic surveys determining the prevalence of *S. neurona* in the horse on reactive bands detected by immunoblot.[45-49] The antigens considered diagnostic in these studies varied. Dubey et al. used 13, 10.5, and 10 kDa.[49] Liang and others reported sera containing antibody to antigens 14 and 16 kDa detected by immunoblot could prevent invasion of *S. neurona* merozoites into cultured host cells.[50] Marsh et al. reported reaction to 29 kDa and 19 kDa proteins when sera and CSF from *Neospora* infected horses were examined on blotted antigens of *S. neurona* indicating the cross-reactivity of serum and CSF antibodies could confound diagnosis.[22] Finally, Rossano and co-workers reported an increased sensitivity and specificity to immunoblotted antigens of *S. neurona* when blots were pre-incubated with *S. cruzi* anti-sera.[51] When *S. cruzi* is used to block cross-reacting antibody in sera and CSF, the antigens of importance were reported as 30 and 19 kDa. These data, taken together, illustrate the confusion surrounding the diagnosis of EPM by immunoblot. Until species specific antigens of *S. neurona* are identified and evaluated the true prevalence of EPM in horses can not be determined. Using the existing parameters the data are confusing and possibly misleading. The consideration of other species of apicomplexan parasites that have reportedly been characterized microscopically or isolated from the spinal cord of horses, *Toxoplasma* and *Neospora*, must be included in any study evaluating EPM in the horse until *S. neurona* specific antigens are fully characterized.

The number of *Sarcocystis sp.* that horses encounter is unclear. To be confident that diagnostic antigens are species-specific antigens, sera from horses that do not contact

S. neurona (i.e., European horses) are needed as a negative control. There is always a trade off in a diagnostic test between sensitivity and specificity. The current test for *S. neurona* infection has a high sensitivity ensuring that neurologically affected horses are differentially diagnosed and treated. Also, the current test for *S. neurona* encephalomyelitis in serum from horses has a low specificity predicting a high prevalence of disease in the equine population. The immunoblot is prepared first by the separation of antigens of *S. neurona* by electrophoresis. The separated antigens are transferred to membranes and the blotted proteins reacted with equine sera or CSF. It is important to note epitopes may be conformational and not detected by SDS-PAGE separated antigens run under reducing conditions. The importance of conformational epitopes has been shown for *S. muris* as well as species of *sarcocystis* that infect sheep.[34,38]

Hypothesis

Despite the importance of a specific assay to detect *Sarcocystis neurona* infection in horses and the cross-reactivity known to exist between sarcocystis species, the important antigens of *Sarcocystis neurona* in equine disease have not been identified and thoroughly characterized. From previous work with other apicomplexan parasites, data suggest that species-specific antigens exist. It is highly likely that this is also true for *Sarcocystis neurona*. We hypothesize that characterization of the antigens of *S. neurona* will identify antigens that elicit an *S. neurona*-specific antibody response. Further, a cDNA expression library from cultured *S. neurona* merozoites provided a genetic basis for evaluation of phenotypic expression of protein antigens. Several major obstacles were overcome to construct and screen the expression library. Novel techniques were required

to isolate and purify the intracellular parasite from the host cells. Studies with monospecific reagents specific for the antigens of interest were needed to confirm species specificity and localization of antigens in the parasite.

Summary

We first determined the *in vitro* growth characteristics of *S. neurona* merozoites isolated from a horse that had histopathologically confirmed EPM in different host cells. We determined that antigens reactive by Western blot and immunoprecipitation did not change detectably with time in culture. We produced polyclonal antibodies to the cultured merozoites of *S. neurona*. We then developed a method for the synchronous release of parasites from host cells. The development and validation of this technique is described in Chapter 2. We then determined which *S. neurona* derived antigens were recognized by infected horses, but not recognized by European horses. These experiments are described in Chapter 3. The location and characterization of a major surface antigen of *S. neurona* are described in experiments discussed in Chapter 4. A monoclonal antibody was prepared against this major surface antigen. The gene encoding the major surface antigen of *S. neurona* was cloned from a cDNA expression library prepared from the mRNA of cultured *S. neurona* merozoites. The antigen was expressed as a recombinant protein in *E. coli* and compared with the native antigen. These accomplishments are described in Chapter 4.

CHAPTER 2

GROWTH, PRODUCTION, AND PURIFICATION OF S. NEURONA MEROZOITES

Introduction

Cyst-forming coccidia of the genus *Sarcocystis* are among the most prevalent parasites of livestock and are responsible for considerable economic losses.[52] Equine protozoal myeloencephalitis (EPM) is a debilitating central nervous system disease of the horse resulting from infection at this site with merozoites and schizonts of *Sarcocystis neurona*. Merozoites of S. *neurona* are obligate intracellular parasites that can be cultured in several host cell lines.[53-55] It is difficult, however, to isolate merozoites in suitable quantity and purity from host cells for molecular studies, because *S. neurona* is a comparatively slow-growing, obligate intracellular parasite and the separation of this parasite from host cell debris is problematic. Further, there are multiple developmental forms of the parasite seen *in vitro* cell culture.[55] Preparing immunoreagents and nucleic acids from this organism requires the most pure sample obtainable.

The calcium ionophore A23187 mediates the exit of mature *T. gondii* trophozoites from infected host cells by increasing the $[Ca^{2+}]$ in the parasitophorous vacuole.[56,57] It is likely that the spike of $[Ca^{2+}]$ transduces the signal that ultimately stimulates exit of the parasite, but this final signal is still unknown.[58,59] The synchronous egress of *T. gondii*

trophozoites from the host cell under this stimulus appears to require complete maturation of the parasite, but parasite release from the vacuole occurs within minutes after addition of the ionophore. Although it has not been observed that *S. neurona* forms a parasitophorous vacuole, the use of this technique for synchronous release of *S. neurona* merozoites and sporozoites was examined.

In this chapter, we have described culture conditions and host cell effects on the growth of the parasite, and the use of the calcium ionophore, A23187, to release parasites from host cells. Parasites thus released were used in antigen analysis. Effects of the ionophore treatment on sporocysts and sporocyst induced primary cultures were also examined. Isolates of *S. neurona* sporocysts obtained from road-killed opossums in Florida were successfully cultured as merozoites in bovine monocytes.

The data identifying and characterizing *S. neurona* antigens is incomplete. Before antigen analysis could be accomplished, several experiments were done. In this chapter, we report the cultivation and purification of *S. neurona* merozoites and the production of polyclonal and monoclonal antisera. We report novel techniques for the manipulation of *S. neurona in vitro*, and further report the characterization of cultured *S. neurona* by electron microscopy and PAS staining.

Materials and Methods

In Vitro Culture of *S. neurona* Merozoites and Host Cell Lines

The UCD1 isolate from the spinal cord from a horse diagnosed with EPM at the University of California was the generous gift of Dr. Antoinette Marsh. This isolate has been maintained in bovine monocyte cells (BM) cultured in RPMI media supplemented

with 10% bovine serum at 37 C in a 5% CO_2/air atmosphere. Established cell lines and primary cultures were maintained in plastic culture flasks incubated under the same conditions and containing media plus serum supplements as described in Table 1. Host cells were released from the culture flask surface by trypsin treatment and transferred to fresh culture flasks at a density of $2X10^4$ per cm^2 resulting in a monolayer that was ~60% confluent. Parasites were immediately added at a density of $2X10^3$ per cm^2. After 3 days of incubation, growth was monitored by counting infective foci using phase microscopy. Parasites were routinely harvested using methods described below when their density approached an average of 5-10 parasites developing within the cells visible in a single microscopic field (400x). The free parasites in the culture medium were monitored by cytospin followed by Giemsa staining.

Studies of the replication of the parasite were performed as follows. Approximately 220 *S. neurona* merozoites recovered from a culture supernatant were added to 5000 Human Lung (HL) cells seeded and growing on Thermonax coverslips in 24 well plates to evaluate the growth and natural release of parasites. Every three days, the 2 ml supernate was removed and evaluated by cytospin and the number of merozoites present counted. The corresponding coverslip was fixed in methanol and stained with Giemsa and the total number of intracellular parasites and those extracellular, but associated with the cover slip, were counted.

Preparation of Sporocysts for In Vitro Culture

Sporocysts of *S. neurona* were obtained by scraping the mucosa of feral Florida opossums that had been killed on the roadways. Mucosal scrapings were stored in

antibiotic media at 4 C until used.[60] *Sarcocystis neurona* sporocyst isolates were selected from among those identified using DNA marker analysis.[27] Prior to being placed in culture, sporocysts were treated in 5% sodium hypochlorite (bleach) for 5 min and washed in tap water by repeated cycles of centrifugation (300 x g, 10 min) and resuspension until the smell of bleach was gone. Sporocysts were floated on an 20/30/60% isosmotic colloidal silica step gradient (Percoll®). Sporocysts obtained at the 30/60 interface were washed twice in PBS, as described above, and stored short term at 4 C. Sporozoites were excysted by either of two methods: 1) ~100 sporocysts were resuspended in 100 µl horse bile containing 2 µl trypsin (5 units/ml) and incubated for 4-6 hours at 37C under a 5% CO_2 95% air atmosphere. Sporozoites and unexcysted sporocysts were collected by centrifugation as above, washed once in phosphate buffered saline, pH 7.2 (PBS) and resuspended in a final volume of 100µl PBS. 2) ~100 sporocysts were resuspended in PBS containing proteinase K (1mg/ml) and 1% SDS and incubated for 10 min. at 37C. Sporocysts were pelleted by centrifugation at 300 x g for 10 min. and washed by resuspension in PBS without additives 3 times. Finally, the pellet was resuspended in 200µl dimethyl sulfoxide (DMSO) freeze media (Fisher) and incubated for two hours to overnight at -20C.

Half of the excysted sporocyst preparation was added to a 25cm^2 flask with a freshly trypsinized, 60% confluent monolayer of BM cells in Dulbecco's medium containing 10% horse serum, 100 units/ml penicillin, 100 units/ml streptomycin, 1mM pyruvate and 1mM glutamate. The culture was maintained at 38 C in an atmosphere of 5% CO_2, 95% air. The culture medium was changed at 24 hours and then at 2-4 day

intervals. Parasite development was monitored by direct microscopic observation with a Nikon inverted microscope at 1-3 day intervals.

Ionophore A23187-Stimulated Release and Parasite Purification

A stock solution of calcium ionophore A23187 (Sigma) was prepared in dimethylsulfoxide to a final concentration of 1 mg/ml and stored at –20C. Infected cell monolayers at 12 days post infection were washed three times with PBS or Hanks Balanced Salts Solution (HBSS), and 10ml of A23187 (1 μM in HBSS) was added to the washed monolayer and incubated at 37C for 40 min. in 5% CO_2, 95% air. Free merozoites were collected by centrifugation and washed in PBS as above. Parasites released by this method were examined by density gradient centrifugation, but further separation from host cell debris by density gradient centrifugation was not routinely necessary. For examination by electron microscopy, parasites were washed in PBS and suspended in 2% glutaraldehyde, dehydrated through alcohol, and embedded in epoxy using standard procedures in transmission electron microscopy by the ICBR Electron Microscopy Core at the University of Florida.

For further purification, parasites recovered from the supernatant were isolated from host cell debris on a discontinuous buoyant density gradient using Iodixanol (Optiprep) in PBS or HBSS. Merozoites were suspended gently in 1.0 ml PBS and were layered onto a preformed, three step discontinuous gradient with layers of 1.03, 1.04 and 1.06 g/ml in a 15 ml round bottom centrifuge tube. The gradient was centrifuged at 1000 x g for 25 min at 20C with the brake off. Fractions containing particulate material at each interface were collected and examined microscopically.

Molecular Analysis of Parasite Cultures

DNA analysis by RAPD markers 33/54 separated the samples into those similar to *S. neurona*, but different than *S. falcatula*, and those that were falcatula-like or 1085-like as described by Tanhauser.[18] Washed merozoites were pelleted by centrifugation for five min. at 16,000g in a microcentrifuge. The pellet was resuspended in 100 µl of lysis buffer (50mM KCL, 10mM Tris-HCL, pH 9.0, 1.5 mM $MgCl_2$, 1% Triton X-100, 1% Tween-20, 1µM Proteinase K), incubated two hours at 56 C, and then boiled for two min. to inactivate the Proteinase K. The tube was centrifuged to remove particulate matter and the supernate was used directly as template in a polymerase chain reaction (PCR). A PCR amplification consisting of the following reagents assembled on ice in a thin-walled microfuge tube: 3µl DNA template, 5µl 10 X PCR buffer, 3µl dNTP mix (2.5 mM each), nuclease free water to 47 µl, 2 µl 33/54 RAPD screening primers. The PCR reaction was 94 C for three min. with hot start, 35 cycles: 94 C 30 sec, 45 C 1 min., 72 C 45 sec, followed by 6 min. at 72 C. The resulting PCR reaction was analyzed on a 1% agarose gel in 1X TBE with ethidium bromide (.5µg/ml) added to the running buffer. These RAPD markers produced a PCR product 1,100 bp. Restriction endonuclease digestions with DraI resulted in a fragment of 884 and 216 bp for *S. neurona*, and digestions with HinfI resulted in fragments 745 and 355 for *S. falcatula*.

Mono Specific Polyclonal Rabbit Anti-sera and Monoclonal Antibodies

A rabbit was immunized at two-week intervals with purified *S. neurona* parasites grown in human lung cells. Two hundred micrograms of protein was combined with Ribi's adjuvant and given subcutaneously in three sites. After three immunizations,

responses of the rabbit against infected cells was determined by a fixed immunofluorescence assay (IFA). The rabbit was rested, boostered, and bled yielding 100 ml serum.

Monoclonal antibodies were produced at the University of Florida Interdisciplinary Center for Biotechnology Research (ICBR) Hybridoma Core Laboratory. All fusions, culturing and hybridoma cloning as well as initial screening were done by ICBR The fusion supernatants were screened by ELISA using antigen prepared from purified *S. neurona* parasites grown in Bovine Turbinate cells. Hybridomas that produced IgG immunoglobulins reactive with *S. neurona* whole parasite lysates but not host cells were screened by Western blot. Antigens of *S. neurona* were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to poly vinyl difluride (PVDF) membranes by standard methods. Single lane strips were cut from membranes and incubated with hybridoma culture supernatants three hours at room temperature or overnight. After washing, membranes were incubated with rabbit anti mouse whole molecule conjugated with alkaline phosphatase. Blots were detected as directed by the manufacturer. All secondary and detection reagents were purchased from Sigma Chemical Company (St. Louis, MO).

Staining

Cells were stained using indirect immunofluorescence as previously described. PAS-McManus' method for glycogen-revised for smears were used to detect differences in surface carbohydrate. The University of Florida Histology core prepared these slides.

Results

Sarcocystis neurona was grown in 11 cell lines to determine growth rate and response to ionophore treatment of the monolayer The results are shown in Table 1. *Sarcocystis neurona* merozoites replicated in two different bovine monocyte lines [BM, laboratory stock culture and BM 0617 (American Type Culture Collection, Rockville, Maryland, USA) CRL 0617], bovine turbinate cells (BT cells, ATTC CRL 1390), human lung cells (HL cells, ATTC CCL 201-8Lu), human foreskin fibroblasts (HFF, ATTC CRL 2450), Chinese hamster ovary cells (CHO cells, ATTC CCL 61), bovine kidney (MDBK cells, ATTC CCL 22), goat tumor cells (GT cells, a gift of Dr. Jack Gaskin), equine dermal cells (ED cells, ATTC CRL 6288), and equine monocytes (EM, primary culture from peripheral blood). Parasite growth in each of these host cell lines was observed over a 30 day period starting from an inoculum of 2000 parasites collected from the culture supernatant of BM cells.

In Figure 1, *S. neurona* is shown growing in bovine monocytes. Phase contrast, A, and geimsa staining, B, preparations represent the typical exuberant growth with rosette formation at five days post infection when sub-cultured from BM 0617 cells. Three densities are illustrated: 1.03 $_{BD}$(arrow head), 1.04$_{BD}$ (star), and 1.06$_{bd}$ (arrow). A. There are two intracellular rosettes and one extracellular parasite shown in this field. With ionophore treatment all merozoites from the rosette would be available for harvest.

Formation of rosettes was first observed 3 days post infection with release and re-invasion of new cells occurring at five days post infection. The relative rate of growth of the UCD1 isolate in each of these host cell lines was estimated by determining the average number of developing intracellular parasites visible per microscopic field

(averaged over 10 randomly selected fields) on day 3 after initiating the culture. These results are summarized in Table 1. Of some interest was the observation that considerably more parasites developed in HL and BT cells than in the line of BM cells from which the inoculum was derived. However, the BM0617 line, was the host cell line best suited for the rapid proliferation of the parasite (See Table 1 and Figure 1). When merozoites were introduced into a new host cell type that sustained growth of the parasite, the replicating forms of the parasite were noticeably larger.

The parasites grew slowly in GT cells and HFF cells producing a large undifferentiated schizont. Electron micrographs revealed that the merozoites did not have prominent micronemes, but contained many electron dense membrane bound structures (data not shown). When propagated in HFF cells, no merozoites were seen outside the host cells until 120 days of incubation, however, detached host cells filled with parasites were seen free in the media. Despite their poor growth in HFF cells, a small percentage of the parasite population remained infectious for BT cells. Parasites transferred from this culture reverted to a normal growth rate and morphology once transferred to a BT cell monolayer.

The efficiency of infection was increased when freshly trypsinized host cells were placed in sufficient numbers in the culture flask to establish a 60% confluent monolayer immediately before merozoites were added. This increase in numbers of merozoites entering host cells improved the yield of parasites and shortened by two weeks the length of time required for culture prior to harvesting the merozoites. Infection of BT cells by merozoites was increased by 50% using scraped cells from thirty day post infection as inoculum rather that the supernatant from the same cultures. This was because the

parasites exited the cells infrequently and asynchronously at 28 to 30 days, and the majority of infectious merozoites remained in the original host cell. By scraping the monolayer, a proportion of the host cells bearing mature schizonts were disrupted releasing additional merozoites that were able to enter other host cells.

Cultures of parasites replicating in HL cells were examined to follow the number of parasites free in the culture supernatant as compared with those found inside host cells during normal parasite growth. These results are represented in a (Figure 2A), graph of extracellular (black bar) and intracellular (white bar) merozoites that were counted every three days post infection of HL cells grown on thermanox coverslips in 24 well plates. The majority of the parasites were released at 21 days in this experiment.

Lung cells at 60% confluence were seeded at a density of 0.1 merozoites per host cell and parasite growth was monitored microscopically for 30 days. Free parasites were not observed until 10 days post infection. During most of the period of observation, the percentage of extracellular parasites was less than 10%. On day 28, the largest number of free parasites was counted; but the next day the numbers of extracellular parasites was back to less than 10%. The observations are summarized in a (Figure 2B) graph showing the number of extracellular parasites in 12 DPI infected monolayers. Duplicate 75 cm flasks were grown for 12 days in HL cells and treated with HBSS or HBSS with 1μM A23187. Free parasites were collected by centrifugation and counted with a hemocytometer

Parasite stages were distinguished by three different buoyant densities 1.03, 1.04, and 1.07 (g/ml) when Iodixanol (Optiprep) was used. The morphology of these forms are represented in Figure 1. The immature schizont that separated with a buoyant density of

1.03 was tear shaped to oblong, had a large round central nucleus with an apparent nucleus:cytoplasm ratio of ~2. The 1.04 schizont was large, rounder, and had a nucleus:cytoplasm ratio of ~1. Early merozoites had a buoyant density greater than 1.06 and usually separated with host cells. This merozoite was long and thin with a small central nucleus and an apparent nuclear:cytoplasmic ratio of ~0.5. This was also the stage seen exiting the mature schizont. Isolation of a pure population of merozoites free of host cell debris was possible using a buoyant density gradient, but this was difficult when host cells detached from the culture flask. Further there was a significant reduction in parasite recovery.

Collection of parasites free in the culture medium was enhanced 90 fold by incubation of infected host cells for 40 min in 1 μM A23187 prior to collecting the culture supernatant. Parasitized host cells that released merozoites in response to ionophore treatment were BM, BM0617, HL, BT, and ED cells. Infected GT, HFF, CHO, BHK, and primary EM cells were refractory for the release of parasites under the same conditions. The release of parasites in response to ionophore treatment was optimal at 10 to 12 days post infection, just after a few parasites were first observed free in the culture supernatant (Figure 2B). Although difficult to accurately determine the percentage of individual merozoites released by this treatment, it was a large proportion. No mature schizonts were visible in a microscopic examination of treated cultures. The mechanism of release was not clear, but the selective disruption of the parasitized-host-cell membrane was seen in electron micrographs. (Data not shown). Initially, the host cell increased in size, became vacuolated, and had small breaks in the membrane. As the plasma membrane became more permeable, empty membrane bound vacuoles or

vacuoles with peripheral ribosomes were released into the media. The host cell became long and cytoplasmic volume decreased. Parasites were observed to move beginning at 10 minutes and continuing until their release at forty minutes. The merozoites undulated hyperactively in this media, but with the removal of the ionophore by addition of culture media, released parasites and the host cells recovered a normal appearance and activity. Ionophore-treated parasites remained animated and readily infected new cells when incubated onto a fresh monolayer. The difference noted in electron micrographs of ionophore treated parasites was that they appeared to have more prominent micronemes than untreated parasites. This can be seen in Figure 3. Figure 4 shows the separation of parasites proteins after they are treated with ionophore, however coomassie blue staining is not sufficient to distinguish parasite antigens from those of the host cells.

Using parasites collected following release by A23187 provided a significant improvement over both of the methods described above for inoculating cultures. Parasites released from the host cell monolayer by A23187 were single, hyperactive, and entered cells readily. When A23187-treated parasites were used as the inoculum, the infected host cells harbored an abundance of mature schizonts in 3 to 5 days. The extracellular parasites remaining after washing off the ionophore did not re-invade ionophore-treated host cells, but increased in size while the few merozoites that remained in host cells formed mature schizonts in five days continuing the infection. Ten days after ionophore treatment, the parasites in the monolayer were unresponsive to calcium ionophore, whereas at thirty days, ionophore treatment again elicited parasite release. During this second ionophore treatment, many host cells were released into the supernatant.

A23187 did not elicit excystation of sporocysts, however the addition of 5% DMSO followed by one freeze thaw cycle did release sporozoites for culture. Sporocyst isolates of *S. neurona* have been successfully cultured in BM cells and propagated for more than 120 days by both methods described (either enzymatic excystation or by freeze-thaw in DMSO). Bovine monocytes were well suited as host cells since phagocytosis resulted in the intracellular localization of sporozoites from excysted and unexcysted opossum sporocysts (data not shown).

Discussion

Cultivation of *S. neurona* in the laboratory requires the infection of host cell monolayers.[2,54,55,60] Multiplication rate differences exist which depend on the parasite isolate, culture conditions, and the host cell.[55] *S. neurona*, strain UCD1, was able to invade eleven different host cell lines but was found to thrive in BM 0617, BM or HL cells. Whereas, after invasion infection was non-progressive in HFF or GT cells. Moderate progressive growth was observed in ED, EM, MDBK, CHO, and BT cells.

Sarcocystis neurona seemed better able to invade cells that were not confluent when placed on a monolayer or preferably added to freshly trypsinized cells. Hermentin and Aspock reported an improved success for the cultivation of *T. gondii* when host cells were infected immediately after sub-culture observing 2-3 fold increase in multiplication rates.[61] They suggested that there was a facilitated invasion of host cells by *Toxoplasma* when the cell's glycocalyx was treated by trypsinization. Also, there was a more far-reaching exploitation of young separated host cells in contrast to cells of confluent monolayers. This appeared to be similar with *S. neurona*, UCD 1. Clearly host membrane components important in invasion by *S. neurona* were not destroyed by

trypsin treatment. All of the changes that take place in the host cell metabolism once the monolayer is confluent are not known, but growth rate ceases and cells eventually grow old and die. *Sarcocystis neurona* UCD-1 thrived in young cells that were actively multiplying and became more static in quiescent cells. It will be important to examine the growth characteristics of other isolates of *S. neurona* comparing these parameters between strains that have been isolated from diseased horses and those derived from opossum feces.

We observed the earliest release of merozoites from mature schizonts at day five after infection of host cells followed by their entry into adjacent host cells and subsequent rosette formation. With rapidly growing host cells, the monolayer became confluent on day four, a day before maturation of the rossettes, whereas the slower growing host cells were not confluent at the first release of merozoites. In the slower host cell lines, BM and HL cells, parasites were seen free in the media sporadically starting at day 10 to 12, indicating that some schizonts had matured. Whereas in the rapidly growing cell lines, MDBK and CHO cells, free parasites were not observed until day 30 or greater. Thus, the rate of maturity of the *S. neurona* parasite appeared to coincide with the metabolism of the host cell. Had they continued to develop and invade new host cells every five days, there would have been an exponential increase in parasite numbers. That was not seen.

The use of calcium ionophore on monolayers that harbored mature schizonts allowed the synchronous release of meozoites with little host cell contamination. The optimum time for parasite release was when a sporadic merozoite was observed in a confluent monolayer from day 10 to 12 post infection. It is possible that use of this

method may also further enhance mass production of parasites especially if it works with the most prolific isolates such as SN6.[62] We show in Figure 4 when SDS PAGE separated proteins of host cells,(lane A); *S. neurona* treated with calcium ionophore, (lane B); and *S. neurona* untreated merozoites, (lane C); are stained with coomassie blue, separated proteins are indicated by the arrows. However, coomassie blue staining is not sufficient to distinguish parasite antigens from those of host cells.

Perhaps the most promising use of this phenomenon may be to synchronize development of the parasite. We found that a homogenous population of highly infective parasites could be released from the host cell monolayer. Little is known about how different developmental forms of *S. neurona* vary antigenically. Dubey found the development of SN6 asynchronous with merozoite-shaped schizonts, immature schizonts, free-floating merozoites, and schizonts present in the culture and suggested the need for standardization of culture conditions to control antigenic variation of cultured *S. neurona*.[62]

The mechanism by which the calcium ionophore stimulates this release is worthy of further study. Apicomplexan parasites possess a calcium storage compartment known as the acidocalcisome. In *T. gondii* when the calcium concentration rises in the parasitophorous vacuole this stimulates microneme discharge and parasite release.[63-65] It is unknown if the release of parasites from the host cell by A23187 is dependent on the presence of a parasitophorous vacuole. *Toxoplasma gondii* is able to invade a wide range of cells in many different hosts by active penetration by the invasive form resulting in the formation of a parasitophorous vacuole. Only the asexual stages of *S. neurona* are known

and are described as residing free in the cytoplasm of tissues from horses and cell culture without a parasitophorous vacuole.[3]

Calcium ionophore did not elicit excystation of sporocysts; however, the addition of DMSO and one freeze thaw cycle did release sporozoites for culture. Bovine monocytes were well suited for initiating cultures from sporozoites. The ability to cultivate *S. neurona* from the sporocyst stage will allow the opportunity to compare parasites at this stage in development with those recovered from equine CNS tissues. Further, it will also allow the comparison of many more isolates. The use of calcium ionophore A23187 was a necessary step to purify merozoites for the identification and isolation of the antigens of *S. neurona* and to produce immunoreagents and nucleic acids for molecular studies.

Host cell lines used in the cultivation of *S. neurona*

Host Cell	Cell Type	#IF/HPF 800X	Response to A23187
1	Bovine Monocyte (BM0617)	5-10	positive
2	Chinese Hamster Ovary	3-5	negative
3	Bovine Kidney (MDBK)	3-5	negative
4	Baby Hamster Kidney	3-5	negative
5	Goat Tumor Cells	1-2	negative
6	Human Fibroblasts	1-2	negative
7	Equine Dermal Cells	3-5	positive
8	Human Lung Cells	5-10	positive
9	Bovine Turbinate Cells	3-5	positive
10	BovineMonocyte Cells	5-10	positive
11	Equine Monocyte Cells	3-5	negative

Table 1. Table showing growth rates (average number of infective foci (#IF)/ten high power fields (HPF), average foci from ten fields counted) of *S. neurona* in indicated cell lines. The response to Ca ionophore A23187 was positive or negative.

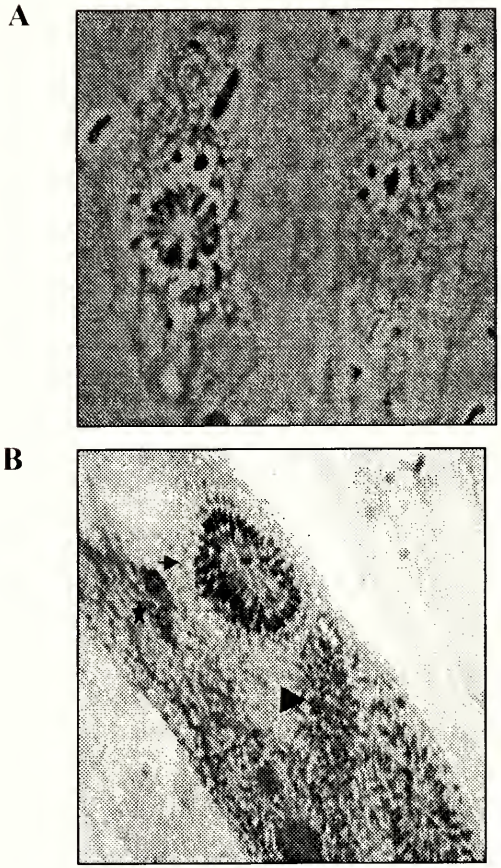
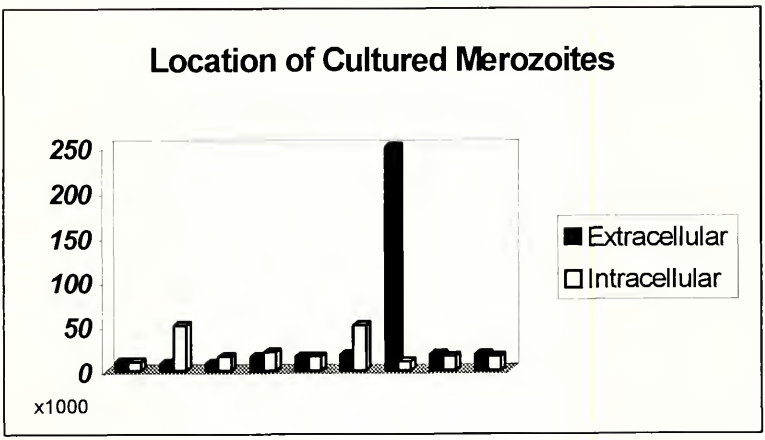


Figure 1. *S. neurona* is shown growing in bovine monocytes Panel A, phase contrast; and Panel B, geimsa stained. Preparations represent the typical exuberant growth with rosette formation at five days post infection when sub-cultured from BM 0617 cells. Three sizes are illustrated: 1.03_{BD}(arrow head), 1.04_{BD} (star), and 1.06_{bd} (arrow). A. There are two intracellular rosettes and one extracellular parasite shown in this field. With ionophore treatment merozoites from the rosette would be available for harvest.

A.



B.

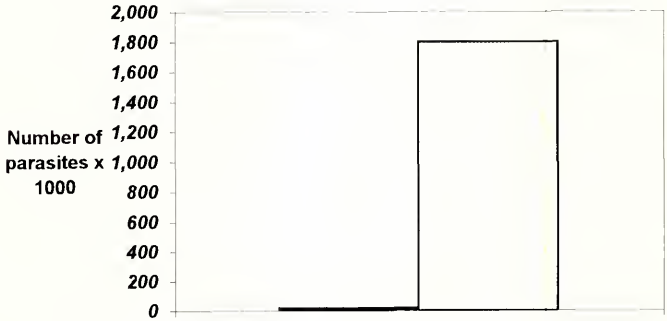


Figure 2. Graphs showing the growth of cultured merozoites of *S. neurona*. A. Graph of extracellular (black bar) and intracellular (white bar) merozoites counted every three days post infection of HL cells. B. Graph comparing the number of extracellular parasites in 12 DPI infected monolayers, untreated (black bar) and A23187 treated (white bar).

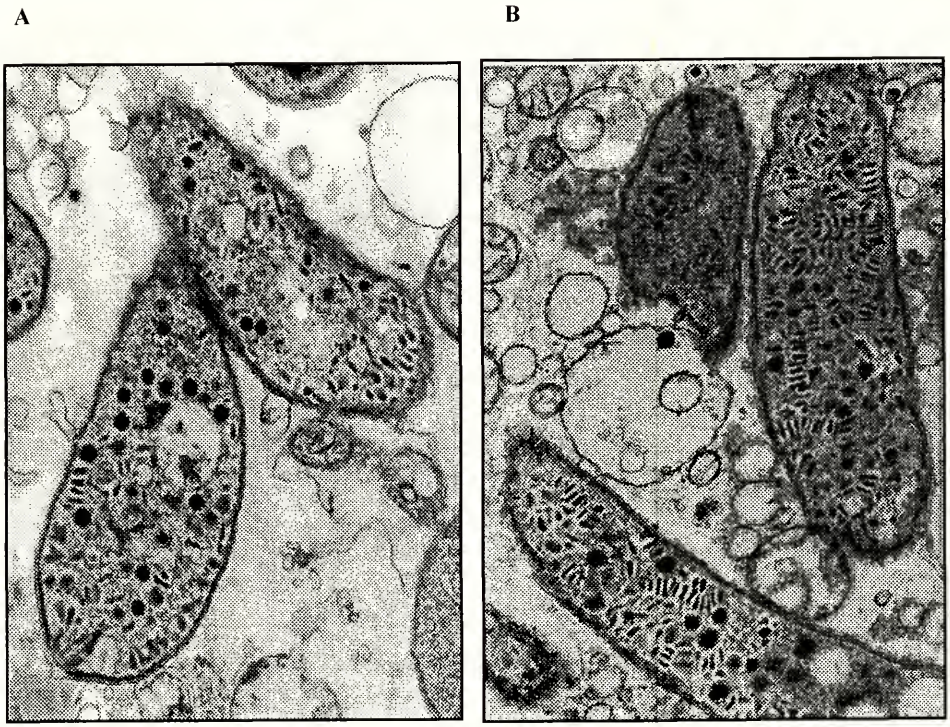


Figure 3. Electron micrograph analysis of cultured merozoites. Panel A, untreated; Panel B, A23187 treated.

CHAPTER 3

IDENTIFICATION SPECIES SPECIFIC PROTEINS OF *S. NEURONA* THAT HAVE DIAGNOSTIC VALUE

Introduction

The name *Sarcocystis neurona* was proposed in 1991 for the apicomplexan parasite isolated from the central nervous system of a horse exhibiting neuromuscular disease.[6] Protozoan parasite infection of the CNS was first reported in horses by Macruz and co-workers and thought to be *Toxoplasma gondii*, even though serum antibodies against *T. gondii* could not be demonstrated.[5] Structurally, *S. neurona* merozoites are similar to, but antigenically distinct from, *Toxoplasma gondii* and *Neospora hughesi*.[4,20,22-23] Horses become infected by ingesting sporocysts of *S. neurona*; although *S. neurona* sarcocysts have not been demonstrated in equine muscle. Antibodies to *S. neurona* merozoites have been demonstrated in serum and CSF from horses with clinical EPM.[63]

During the last ten years, molecular tools have been used to investigate the life-cycle and identify the sporocyst stage shed by the putative definitive host, the opossum (*Didelphis virginiana*).[19,24,30,67] Despite recognition of the infectious stage of *S. neurona*, experimental infection of horses has been disappointing. Seroconversion of horses

challenged by administration of sporocysts from the opossum has been demonstrated by immunoblot, but the organisms have not been recovered from the central nervous system of these animals.[28,30] DNA methods have been developed to differentiate between several sarcocystis species carried by the opossum.[19,24] The experimental infection trials neglecting the molecular characterization of sporocysts used for challenging horses might be subject to error. This possibility for error must be recognized in interpreting data concerning seroconversion in experimentally infected horses.

All that is known about equine *S. neurona* sarcocystosis comes from natural exposure. Post mortem examination demonstrating organisms in histological lesions or recovery of the organism by culturing the etiologic agent is required to establish a definitive diagnosis. Unfortunately, there are no current methods for the conclusive diagnosis EPM pre-mortem. Fayer and co-workers performed a retrospective study based on the presence of protozoa in CNS tissues from horses to determine the epidemiology of EPM in North America.[67] Since that time, the estimates of prevalence of infection in the horse have been based on serum Western blot test results made commercially available by Granstrom.[45-49] Because of the high sero-prevalence of *S. neurona* antibodies detected in horses, *S. neurona* has acclaimed significance as an infectious disease of horses in the Americas. However, immunobloted proteins considered indicative of the presence of specific antibody vary.[45-49]

Similar antigens shared by several *Sarcocystis* spp., as well as *S. neurona*, are well documented.[2,7,12,33,34] Initial work with *S. neurona* involved studies that compared reactivity of this "new" organism to that of *T. gondii* and other *Sarcocystis* sp., such as *S. fayeri* and *S. cruzi*, using serum antibodies from natural infections or raised antibodies.[12]

When hyperimmune anti-*S. cruzi* bradyzoite antibodies were used to detect *S. neurona* organisms in clinical tissues, *S. cruzi* antibodies were found superior to antibodies directed against *S. neurona* merozoites.[68] Based on these observations, cross-reactive antibodies were exploited using avidin-biotin complex (ABC) immunoperoxidase methods. The first antemortem EPM test available was an IFA test that employed *S. cruzi* as antigen for detecting *S. neurona* antibodies. Due to cross-reactive antigens detected, results are reported as a non-specific *Sarcocystis* titer.[68]

More sensitive tests were developed, however cross-reactivity of antibodies against *Sarcocystis* sp. that contact horses confounds the interpretation of these test results. Descriptions of the commercial Western blot test developed in 1993 reported serum and CSF antibody reactivity to 22kD, 13kD, and 10.5kD immunoblotted proteins as indicating exposure to *S. neurona*.[12] The same investigators experimentally challenged horses and reported that antibody reactivity to 13kD, 11kD, 10.5kD and 10kD blotted *S. neurona* proteins were diagnostic.[30] Alternatively, Marsh and co-workers indicate proteins of 29kD and 17kD are *S. neurona* specific.[22] Rossano used sera from true positive (histopathologically confirmed cases of *Sarcocystis* infection) and true negative horses (not infected with *S. neurona*) to define the association of infection status with antibodies and found reaction to 30kD and 16kD bands, when separated by SDS gel electrophoresis using 12-20% gradient gels, were consistent with known disease status.[51]

The successful identification of species-specific antigens will advance the detection of diagnostic antibodies present in equine serum and CSF. The accuracy of test interpretation when evaluating the reactions of sera and CSF fluids will increase when there is an understanding of the antigens identified by antibodies induced by *S. neurona*

infection. Additionally, the elucidation of the molecular nature of specific antigens will help define which antigens have potential diagnostic value. Lack of knowledge concerning the identification of the antigens of *S. neurona* recognized by immunoblot tests requires careful interpretation of test results. Seroprevalence estimates indicate high rates of infection in clinically normal horses. This observation is supported by Rossano because all the sera tested (N=57) from horses in the Eastern hemisphere (where opossums are absent) demonstrate antibody to 62kD, 30kD, 16kD, 13kD, 11kD, 10.5kD, and 10kD bands.[51] Several explanations are supported by these data. These observations could be due to errors in interpretation due to cross-reactive antigens, antigens shared by species of *Sarcocystis* that use the horse as an intermediate host. Alternatively, more than one protein could migrate to a specific band on a 12-20% linear gradient single dimension gel. Because data gathered and published to date base interpretation on an antibody response to undefined proteins, these interpretations are incomplete. A more accurate detection of *S. neurona* antibodies will require more precise definition of the proteins to which they react. When species specific proteins are defined, then more precise estimates of seroprevalence and the risk factors associated with infection can be made.

Sarcocystis neurona can be cultured in the laboratory using techniques previously developed for *in vitro* culture of *S. cruzi*.[2,68-69] Work done by Granstrom and co-workers has suggested that *S. neurona* is morphologically different from other species of *Sarcocystis* that infect the horse.[6,7,12] *Sarcocystis neurona* merozoites represent an excellent source of parasite derived proteins to study the antigens that are important in the host immune response as well as identification of proteins that could improve the

diagnosis of EPM.

Rabbit polyclonal antibodies have been raised against *S. neurona* merozoites and these antibodies evaluated using indirect fluorescent antibody testing and immunohistochemistry.[68] *Sarcocystis cruzi* anti-bradyzoite serum was a better reagent than antibody raised against *S. neurona* merozoites for the detection of *S. neurona* merozoites in tissues of naturally infected horses.[68] The differences between the reactivity of *S. cruzi* and *S. neurona* antisera results may be related to the antigenicity of the different stages of sarcocystis (bradyzoites vs merozoites) that induced the antibody response in the rabbit. Recently, Rossano and co-workers reported that *S. cruzi* antibody blocked blotted proteins not specific to *S. neurona* resulting in an increased sensitivity and specificity of the test when the blot was reacted with equine serum.[51] The *S. cruzi* antibody used by Rossano was derived from cattle that had a positive test result for *S. cruzi* when screened by IFA.

Sommer and co-workers used two-dimensional electrophoresis to show cross-reactions between *S. muris* and *S. suicanis* proteins in two developmental stages indicating inter-species as well as interstage cross-reactivity.[35] Tenter was able to separate antigens from several *Sarcocystis* species using chromatofocusing demonstrating species specific antigens and suggested their importance in immunodiagnosis.[34] Extensive analysis of *T. gondii* antigens recognized by infected hosts has been made elucidating the hosts' humoral immune response and has served as a basis for the immunodiagnosis of toxoplasmosis.[70] The presence of stage-specific epitopes as well as epitopes shared by stages of *T. gondii* have been demonstrated and used to study the transition between developmental stages.[70-72]

There are several reported factors that affect the transition from tachyzoite to bradyzoite stages in *T. gondii* such as temperature, pH, mitochondrial inhibitors, and sodium arsenate.[73,74] Also, it has been suggested the host's immune system may play a significant part in directing the course of infection in *T. gondii* infections.[70] Radchenko fed mice a single dose of *S. muris* sporocysts and observed that the number of sarcocysts per animal increased regularly as the time of infection was progressing, being higher after six to ten months post infection than at one to four months.[75] This phenomenon was explained with electron micrographs showing, along with numerous normal sarcocysts, the presence of some separate, individual zoites both within and outside muscle fiber, in the endomysium. Interestingly, Jakel and co-workers reported the synchronous development of two morphologically, cytochemically, and immunologically distinct types of merozoites of a *Sarcocystis* spp. during its asexual phase in the intermediate host.[76] Multiple infections must be considered in all infections derived from natural exposure to feces from a host that can harbor more than one sarcocyst species.[18] Because cross-reactive antigens, stage variable antigens, and co-migration of proteins in SDS gels are known to occur, it is important to use caution and extensive analysis when characterizing diagnostic antigens for detection of sarcocystis by immunoblot. With the information available today, the interpretation of antibody test results are confusing and limited.

Based on immune sera from seven histologically confirmed cases of EPM, one horse inoculated with *S. neurona* merozoites, one pony with *S. fayeri,* and immune sera from rabbits inoculated with *S. cruzi* bradyzoites, *S. muris* bradyzoites, and *S. neurona* merozoites, Granstrom proposed potential candidate proteins for the development of an *S.*

neurona specific immunoassay.[12] In 1993, Granstrom based his commercial immunoblot on SDS-PAGE separated antigens of cultured *S. neurona* merozoites.[43] He found 104 proteins that ranged in size from 14 to 300 kDa were recognized by most of the limited sera tested. He noted eight proteins that were detected by sera from EPM-affected or *S. neurona*-inoculated horses that were not recognized by pre-or post-infection sera from the *S. fayeri*-infected pony or pre-inoculation serum from the *S. neurona*-inoculated horse. He also found six of these proteins were not recognized by rabbit anti-*S. cruzi* or *S. muris* sera. Additionally, Granstrom concluded serum IFA titers could not clearly differentiate between *S. fayeri* and *S. neurona* and considered specific reactivity to blotted proteins at 22kD, 13kD, and 10.5kd by antibodies in equine serum and CSF as evidence of exposure to *S. neurona*. By 1997, a different criterion was used by the same laboratory when they published the results of a horse challenge study.[30] In the challenge experiment reactive antibodies to proteins at 13kd, 11kd, 10.5, and 10kD were considered a positive test result. The dilution of immune sera, chronology of the disease, sporocyst dose, length of exposure, dissemination of merozoites, treatment regimen, and stage of parasite eliciting an immune response could have affected the observed results.

In a later retrospective study, Liang observed four common immunoblot patterns when data from 25,000 sera and CSF samples were examined by the commercial test.[50] No correlation was made between immunoblot pattern and disease, however the immunoblot pattern was correlated with ability to neutralize infection in cultured bovine turbinate cells. Liang further suggested two proteins, 14kD and 16kD were on the surface of the parasite and were responsible for neutralization of infection.

Other independent studies indicate different antigens are important for detection of *S. neurona* in the horse. Rossano defined reactivity to 30kD and 16kD bands of separated *S. neurona* proteins as the criterion for a positive test when equine sera was tested using blotted proteins blocked with *S. cruzi* antisera.[51] Another apicomplexa has recently been recognized as an etiologic agent in encephalomyelitis in horses.[22,23,49] In natural infections the number of sera from *Neospora* infected horses available for examination are limited. It has been recognized in recent work by Marsh et al. that *S. neurona* infections may be mis-diagnosed if *Neospora* is not considered as a diagnosis in EPM and that careful intepretation of Western blot data using antigens of *Neospora* and *S. neurona* are necessary. Marsh et al. indicated that immunodominant proteins of 29kD and 17kD are specific for *S. neurona* and differentiated natural infections of *Neospora* and *Sarcocystis*, both species that can infect the CNS of horses.[22]

The data identifying and characterizing *S. neurona* antigens is incomplete. Before diagnostic criteria are suggested for the interpretation of serum and CSF antibodies it is important to define antigens that are *S. neurona* specific and immunoreactive to antibodies in equine serum and CSF recovered from diseased animals. The experiments defining *S. neurona* specific antigens were done. In this chapter I report the identification of *S. neurona* antigens, indicating the molecular weight and pI, by 2D electrophoresis. I describe the development of specific antibody to these antigens and demonstrate their reaction to blotted *S. neurona* antigens. We show the location of these antigens in whole parasites using IFA and postembedding immunogold labeling. Finally, we characterize the antigens identified as *S. neurona* specific that are identified by CSF from a horse with EPM and demonstrate their presence in clinical tissue.

Materials and Methods

In vitro Vitro culture Culture of parasitesParasites, antigen Antigen Preparation

Sarcocystis neurona UCD1 strain merozoites isolated from a horse with EPM were grown and maintained in bovine turbinate cells (BT cells, ATTC CRL 1390, American Type Culture Collection, Rockville, Maryland, USA), or human lung cells (HL cells, ATTC CCL 201). Confluent 150 cm^2 flasks of BT cells that were grown in 10% (v/v) fetal bovine serum (FBS) in DMEM and supplemented with 100 U penicillin G/ml, and 100 µg streptomycin/ml, (growth media), were trypsinized, divided, and seeded with 5X 10^5 merozoites. Cell cultures were incubated at 37 C in a humidified atmosphere containing 5% CO_2 and 95 % air. Media was added thrice weekly. Antigen for animal inoculation and ELISA screening was prepared by removing free merozoites from 28 day old cultures and placing them on a 60% confluent monolayer of HL cells. The parasites were maintained in HL cells for twelve days. Parasites were collected from the media and washed in PBS containing a mixture of protease inhibitors (Boehringer). Parasites were resuspended at a concentration of 1 mg wet weight cells/ml then frozen/thawed to disrupt cells. To release cells with ionophore infected monolayers were washed three times with Hank's balanced salt solution. A working solution of 1 µM calcium ionophore (Sigma A23187) was made by dilution in PBS or HBSS. Ten ml working solution of ionophore was added to a 150 cm^2 flask and incubated at 37 C until parasites were released from cells, about 40 min.. This antigen was sent to Lampire biologicals for the production of polyclonal rabbit antiserum and the University of Florida Hybridoma Core for the production of monoclonal antibodies. For all immunoelectron microscopy (IEM) work,

pre-immune mouse and IgG isotype controls were used using IgG human lymphoma monoclonal (523) that was provided as a standard control by the the University of Florida Hybridoma Core. *Neospora caninum, Neospora hughesi*, and *Toxoplasma gondii* were grown and maintained on bovine monocyte cells (BM cells, ATTC 618) Antigens from *Neospora* and *Toxoplasma* were prepared as described for *S. neurona.*

Antibody production

Rabbit antibodies were produced as described in Chapter 2. Additionally, two mice were immunized with *S. neurona* parasites harvested from 12-day-old cultures that had been treated with calcium ionophore to release merozoites from host cells. The third mouse was immunized with protein containing gel pieces cut from NuPage gels in the region corresponding to p30. On initial screening mouse 3 had a low serum titer to *S. neurona* detected by ELISA and was boostered with killed whole parasites. For hybridoma production the spleen cells from mouse 3 was added to spleen cells harvested from mouse 2. Spleen cells from mouse one were prepared separately from the combined cells from mice 2 and 3.

Western Blotting

Parasites for immunoblot were grown in BT cell cultures for 12 days and parasites released as described above. Ten million merozoites were harvested for SDS PAGE analysis. Harvested merozoites were washed in PBS containing protease inhibitors (Boehringer) and resuspended in 4X LDS solubilizing buffer (Novex) to yield 2.5 mg/ml total protein. The sample was vortexed, heated to 70 C for ten min., and centrifuged in a microfuge to remove insoluble material. Ten µl was loaded per lane. Uninfected host

cells were treated the same way and used as a negative control. Immunoblotted antigens were separated using 4-12% Bis-Tris NuPage Gels (Novex) and blotted to PVDF. Blotted antigens were incubated in primary antibodies and washed 3 times in 300 volumes of blocking solution (1% BSA in PBST with 1% sodium azide). The washed blots were placed in alkaline phosphatase conjugated secondary antibody and incubated for one hour at room temperature. The blot was washed in blocking buffer and detected with *p*-nitrophenol phosphate substrate tablets (Sigma). Additionally, monoclonal antibody diluted 1:500 was sent to EBI, a commercial laboratory, and run against their isolate to enable comparison between isolates.

Periodate and Phospholipase C Treatment of *S. neurona*

Parasites were harvested as above and incubated in PBS containing 50 mM periodate for one hour prior to being processed for electron microscopy at the EM Biotechnology Core (UF). For the ELISA, Nunc Maxisorp plates were coated with 50 µg *S. neurona* antigen or bovine enterokinase (positive control) and incubated overnight. Plates were washed four times and blocked with 1% BSA in PBS with 1% sodium azide. Plates were washed five times and then filled with 300 µl sodium acetate. Freshly made periodate at a starting concentration of 50mM was serially diluted in sodium acetate giving a final well volume of 100 µl. Plates were incubated for one hour, in the dark, at room temperature. Plates were then washed once with 300 µl 50 mM sodium acetate, pH 4.5, soaked for 5 min. and the solution aspirated. Wells were filled with 100 µl 1% glycine and soaked for 30 min. at room temperature and then washed five times. The ELISA then was continued with the addition of primary antibody, mass culture

hybridoma 4F1, at 0.5μg/ml and 0.25μg/ml. Bovine enterokinase was used as a positive control with HL 523 monoclonal antibody (isotype control) that recognizes a carbohydrate epitope on bovine enterokinase or media (negative control) as a primary antibody. Preparations of whole cell antigens of *S. neurona* were blotted to PVDF membranes (as described for Western blotting) followed by a 60 minute incubation in blocking buffer. Blots were incubated with from 0 to 50 mM periodate, washed, and processed as described for immunoblotting.

Freshly harvested parasites for phospholipase C treatment were washed and resuspended in PBS, pH 7 to which 1 U phosphatidylinositol phospholipase C was added. Tubes were incubated one hour at 37 C and then processed for IEM or SDS-PAGE.

Parasites were treated for ten min. to one hour in 0.25% trypsin-EDTA and submitted for IEM.

ELISA protocols

Standard ELISA protocols were used. Briefly, Nunc Maxisorp plates were prepared by the addition of 50 μl *S. neurona* antigen or host cell antigen diluted to 10μg/ml in carbonate-bicarbonate buffer and incubate overnight at 4 C. The plates were washed 4 times and blocked in blocking buffer, 1% bovine serum albumin in 1x PBST (1X PBS, 0.05 Tween 20, 1% sodium azide) and incubated for 60 min. at room temperature. One hundred microliters of primary antibody at several dilutions (4F1 mAb, mouse pre-immune serum, IgG, 1% BSA without serum, or media) was incubated for 1 hour at room temperature followed by four washes in blocking buffer. A commercial rabbit anti-mouse whole molecule (Sigma A 1902), goat anti-mouse IgG γ chain specific(Sigma A3438), or goat anti-mouse IgM μ chain specific (Sigma A 9688) alkaline

phosphatase conjugated secondary antibody was added and incubated for 60 min., washed three times with PBST. followed by the addition of para-nitrophenyl phosphate. The OD was measured and recorded at 492 nm at 30 and sixty min..

IFA

Standard IFA protocols were used, but fixation of the parasites varied. *S. neurona* was grown in BM cells on glass slides that were coated with collagen followed by fixation in methanol or formalin. Parasites were harvested from 150 cm^2 flasks and washed with PBS and used fresh or prepared by cytospin and fixed with methanol or formalin. Slides were blocked with the application of 100 µl dilute goat serum for 30 min. at 37 C to block non-specific binding. Slides were washed in PBST bath for 5 min. with three changes of buffer, drained of excess fluid and primary antibody (100 µl 4F1 at 1:1000, rabbit anti-*S. neurona* polyclonal antisera, or pre-immune mouse anti-sera) was applied and incubated at 37 C for 1 hour. Slides were washed, drained, and incubated with an appropriate secondary conjugated antibody and mounted with DAPI.

Immunoprecipitation and 2D Electrophoresis

Sarcocystis neurona was grown and washed as described above followed by one wash in carbonate buffer (50mM Na_2CO_3, 0.85% NaCl, pH 7.4) and centrifuged X300 g for 10 min.. Merozoites were then washed twice in Na_2CO_3 buffer at 4 C and placed in lysis buffer (50mM Tris-HCl, pH 7.6, 1% TritonX-100, 1mM EDTA) at 4C for 30 min.. Parasite lysate was centrifuged at 3000 X g for 30 min. at 4C. The pellet was resuspended in 2DE (two dimensional electrophoresis) sample buffer (8M urea, 100 mM dithiothreitol, 4% CHAPS, 0.5% carrier amphylolytes pH 3-10, and 40 mM Tris-HCl).

The lysate was *q.s.* to 1 ml with dilution buffer to which 10 µl polyclonal rabbit anti-*S. neurona* serum was added and the mix was incubated 30 min. at 37 C. After incubation 150 µl washed (lysis buffer) GammaBind Plus Sepharose gel (Pharmacia) was added and incubated for 30 min. at 4 C with shaking. The gel was washed by centrifugation in a microfuge twice with lysis buffer and once with water (to remove salt) and 135 µl 2DE sample buffer was added and held at room temperature for 30 min. followed by centrifugation at 3000 g 30 min. The supernate was removed to a new tube and centrifuged at full speed in a microfuge for 5 min. to remove any remaining gel.

A pH 3-10 IEF (Bio-Rad) strip was rehydrated overnight with 125 µl of sample followed by isoelectric focusing with a step gradient: 200 V (.01H), 3500V(1.5H), 3500V(2H). Strips were equilibrated in SDS buffer (2 % SDS, glycerol, BME, 6M urea) for 15 min at room temperature, separated on 4-12% Bis-Tris gels and blotted.

Results

One rabbit was immunized with *S. neurona* whole cell antigen and developed high titer anti-*S. neurona* antibodies. *S. neurona* merozoite total cell protein separated by SDS PAGE is shown in Figure 5. Coomassie blue stained proteins in Lane A are compared to antigens blotted onto PVDF membrane and probed with polyclonal anti-*S. neurona* antibodies in Lane B. The rabbit antibodies recognized a group of antigens indicated by the arrows. Notable is that the antigens at approximately 35 to 28 kDa are recognized by immune sera as five bands on this gel system. Generally a broad band is recognized at approximately 30 kDa when gradient gels are used. This can be seen in later figures (Figure 8).

Three coomassie blue stained gels demonstrate the reproducibility of carbamylated protein standards focused using IPG strips and separated with SDS PAGE in the second dimension, data not shown. First, the reproducibility of 2D electrophoresis using IPG strips was determined when the distance of migration in mm (Y axis) was compared to the molecular weight of the protein. The reproducibility of 2D electrophoresis using IPG strips was determined when the distance of migration in mm (X axis) was compared to the pI of the protein. Finally, the approximate pI of *S. neurona* proteins separated by 2D electrophoresis was determined from comparison with carbamylated protein standards, see Figure 6. Arrowheads mark approximate pI when compared to standards. The standards are from left to right: amylogulcosidase, 89 and 70 kDa, pI 3.8; ovalbumin 45 kDa, pI 5.1; carbonic anhydrase 29 kDa, pI 7.0; myoglobin 17 kDa, pI 7.6 . It is not possible to determine which proteins are derived from host cells on this coomassie blue stained gel. In an effort to determine which antigens were parasite, we examined immunoblots probed with antibodies from rabbit anti-*S. neurona* immune sera. Also, we used serum and CSF from horses with histopathologically confirmed EPM to determine which antigens are recognized by diseased horses.

CSF fluid from a horse with EPM was used to probe *S. neurona* antigens that were separated in two dimensional gels, blotted to PVDF membranes. The antigens of *S. neurona* recognized by CSF from a horse diagnosed with EPM are demonstrated in Figure 7 A. These antigens identified by equine antisera were compared with those detected when similar blots were probed with antibodies from a hyperimmunized rabbit, Figure 7 B.

In an effort to elucidate the difference in antigens recognized by equine CSF and polyclonal rabbit anti-*S. neurona* sera, further experiments were done. Results of these experiments are shown in Figure 8. These antigens are immunoprecipitates separated by SDS-PAGE on a 4-12% Bis Tris gradient gel. The most outstanding observation is that a horse with histologically confirmed case of EPM can precipitate the 30 kDa antigen that is detected by the rabbit antisera, (B, lane 1) but does not recognize this as the major antigen on a blot when probed with equine CSF. Likewise, the rabbit antisera precipitated the 19 kDa antigen, (A, lane 2) but recognized the 30 kDa antigen as the predominant antigen. It is interesting that although the rabbit antisera does not detect the 19/17 kDa antigen as dominant, when used to probe CSF immunoprecipitated antigens (B, lane 1 and 2), the rabbit antisera was a better capture reagent than the equine CSF (A, lane 2 versus A, lane 1). The polyclonal rabbit anti-*S. neurona* antibodies were then used to concentrate the antigen by immunoprecipitation. An immunoblot of immunoprecipitated *S. neurona* antigens detected with CSF fluid from a horse with histopathologically confirmed EPM is shown in Figure 8A, lane 2. The same blotted antigens detected with polyclonal rabbit anti-*S. neurona* antisera, Figure 8B, lane 2. The horse CSF antibodies percipitated antigens that separated at approximately 28 kDa, Figure 8A and B lane 1, however, the major antigens recognized by the horse were at 19 kDa. The rabbit recognized major antigens at 29 kDa.

One mouse was immunized with *S. neurona* whole cell antigen and responded with an ELISA titer of greater than 1:8,000 to *S. neurona* cultured merozoites, Figure 9. These charts show ELISA data for a mouse immunized with solubilized antigens of *S. neurona* merozoites. Antibodies to whole mouse immunoglobulin molecule serum levels

show a good response to *S. neurona* but not host cells, Figure 9A. The antibodies were determined to be IgG, (Figure 9B). No appreciable titer to host cells was observed before or after immunization. Screening of hybridomas yielded a mass culture supernatant, 4F1, which reacted by ELISA at greater than 1:16,000 and reacted on immunoblotted whole cell *S. neurona* antigens to a single band but not to host cells. In Figure 10A SDS-PAGE separated antigens of *S. neurona* were reacted against hyper immune mouse serum prior to fusion. Figure 10B shows the reaction of mass culture antibodies, 4F1, after fusion and one round of cloning, demonstrating the reaction to a 29-30 kDa antigen of cultured merozoites. The mass culture antibodies were used to probe antigens of *S. neurona* cultured merozoites two spots, 29 kDa were prominent (Figure 10C).

The 4F1 antibodies did not react to host cells, *Neospora* or *T. gondii* by immunoblot (data not shown). The antibodies of mass culture 4F1 were used in an ELISA to determine cross-reactivity with two Apicomplexan parasites known to infect horses. The graph of the data is shown in Figure 11A and indicates that 4F1 does not bind *Neospora hughesi* merozoites, *Toxoplasma gondii* tachyzoites, or host cells. In the assay the *T. gondii* antiserum was not specific for *T. gondii*, and the assay was repeated. The results are shown in Figure 11, B. Antibody 4F1 bound a 29 kDa antigen on blots of merozoite whole cell extracts. A band of similar size is also recognized by mouse polyclonal anti-*S. neurona* antibodies. The 4F1 antibody preparation also recognized *S. neurona* antigens immunoprecipitated by CSF from a histopathologically confirmed case of *S. neurona* EPM (compare Figure 10 to Figure 7A and Figure 7B). 4F1 antibody preparation was used to probe *S. neurona* native antigen separated by 2D electrophoresis and detected a protein that migrated as two spots, Figure 10 C.

The 4F1 antibody preparation reacted with live cells or formalin fixed *S. neurona* but not host cells. The binding of 4F1 antibodies to the surface of formalin fixed parasites is shown in Figure 12 A. Similar results were obtained with methanol fixed parasites (data not shown). The phase contrast image of the same field, Figure 12 B demonstrates that there is no binding to host cells. The controls for this experiment, pre-immune mouse serum and isotype control, also showed no binding to *S. neurona* by immunofluorescence assay, (data not shown). Post-embedding immunogold labeling of *S. neurona* cultured merozoites using 4F1 antibodies also shows that the surface of the parasite is labeled, Figure 13 A. Controls included a mouse monoclonal isotype control, Figure 13 B. The cloned hybridoma 1631, derived from 4F1, was used to label the surface of *S. neurona* cultured merozoites by post-embedding immunogold labeling and is shown in Figure 13 D.

Although it is possible that the purified 4F1 antibodies could be a mixture of two or more antibodies (because the sub-cloning had not been completed when these antibodies were purified) it is probable that 4F1 was a single clone. The antibodies were purified from the supernate obtained from a first sub-cloning step in the isolation of a hybridoma from a mouse spleen fusion, these wells were seeded by limiting dilution. The antibody preparation 4F1 was identified as IgG-1, and it identified one band when used to probe immunoblots of native *S. neurona* antigens. The purified antibodies reacted specifically with the surface of the parasite by electron microscopy and IFA. Finally, only one monoclonal antibody was obtained from this mass culture preparation upon further cloning and when experiments were repeated with "sister" clones, the results were identical to those obtained from the 4F1 mass culture antibodies. These observations,

when taken together indicate 4F1 antibody preparations were monoclonal in nature. Although the initial antibodies from the mass culture were probably from a single clone, and therefore represent a monoclonal antibody another possibility exists. It is also possible that this monoclonal antibody recognizes either two distinct proteins instead of just one, or two distinct pools of the same protein (perhaps one glycosylated and the other not). The data supporting this statement is that the band detected when 4F1 monoclonal antibodies are used to probe native *S. neurona* proteins separated by SDS-PAGE is diffuse; when *S. neurona* merozoites are treated with trypsin followed by SDS-PAGE and immunoblotted the band is not diffuse (data not shown). It is possible that the fuzzy band represents a glycoprotein, the epitope recognized on live cell IFAs is probably present only on the external surface. Thus, cleaving with trypsin eliminated the reactivity with the fuzzy band on the western blots, and continued reactivity is not seen with a smaller band as it might if the epitope were also present on a cytoplasmic domain. Post embedding immuno-gold labeling data supports these immunoblot observations because labeling was maintained on the surface of the parasite after trypsin treatment (data not shown). After trypsin treatment, additional binding of 4F1 antibodies to internal structures was observed by post embedding immuno-gold labeling. The interpretation of these results is difficult without further experiments to elucidate whether reactive epitopes on different proteins were exposed by the trypsin treatment or if epitopes on newly synthesized protein was available for reaction.

Sarcocystis neurona merozoites were phospholipase C-treated to determine if the epitope that was detected by the 4F1 antibody preparation was on a glycolipid anchored protein. The results indicate the label is not removed by phospholipase C treatment

indicating that the antigen is not glycolipid anchored (Figure 13 C). Isotype monoclonal antibody controls showed no binding to the merozoites, (data not shown).

When parasites were treated with 50 mM periodate and examined by IEM, the binding of 4F1 to the pellicle was unaffected (data not shown). The epitope recognized by 4F1 was not removed with periodate treatment as shown by ELISA in Figure 14. This chart shows the data obtained by ELISA to determine the nature of the epitope detected by 4F1 mass culture. Periodate did not remove the epitope recognized by 4F1 while the carbohydrate epitope recognized by a monoclonal antibody to enterokinase was removed with periodate treatment. The same results were obtained by immunoblot when *S. neurona* antigens were separated by SDS-PAGE and the blotted proteins treated with periodate, 4F1 binding was not removed with increasing concentrations of periodate from 0 to 50 mM (data not shown). Similarly, post embedding immuno-gold labeling was not removed from merozoites treated with 50mM periodate for thirty min. at 25C (data not shown).

The cultured merozoites of *S. neurona* were used in an immunoprecipitation assay to concentrate antigens to be used in immunoblotting experiments. When 1% triton was used to solublize antigens, the insoluble pellet contained proteins detected by 4F1. They were separated by 2D electrophoresis and probed with 4F1 antibodies detecting a smear at 29 kDa, Figure 15 A. The form of the material found in the insoluble fraction was examined by electron microscopy and determined to be to the cytoskeleton of the parasite Figure 15 B.

The 4F1 antibodies were sent to a commercial laboratory (EBI, University of Kentucky) and included on their immunoblots in an effort to determine whether or not the

4F1 antibodies bound other isolates of *S. neurona*. In Figure 16 the arrow marks the lane developed with 4F1. It detects a 28 kDa antigen from the isolate used in the commercial immunoblot test. A 28 kDa antigen is also detected by equine serum and CSF samples on the samples on the same blot.

Rabbit anti-*S. neurona* antisera and 4F1monoclonal antibodies were also used to probe clinical tissues for the identification of *S. neurona*. Figure17 shows the results when 4F1 antibodies were used to detect *S. neurona* in the spinal cord of a horse with clinical signs of EPM. The hyperimmune rabbit antisera also identified merozoites in clinical tissue (data not shown). Both reagents clearly identified parasites in host tissues that were otherwise identified ambiguously when observed by hemosin and eosin stain. .

The mass culture 4F1 was examined in a competitive ELISA to determine the use of this assay in the measurement of antibodies in clinical samples. Figure 18 shows the results of the assay using 4F1 in a competitive ELISA that measured the percent of inhibition of 1 microgram/ml of 4F1 by equine serum and CSF. Although serum did competitively inhibit binding CSF did not. The media used to prepare monoclonal antibodies was also found to compete for the antibody binding site.

Discussion

The production of specific antibodies will be important tools enabling the characterization of specific antigens of *S. neurona*. The antibody 4F1 (isotype IgG1) was produced and recognized an apparent 29 kDa antigen, pI 7.3, of *S. neurona*. It did not recognize antigens of *T. gondii or Neospora*. These antibodies bound to an epitope that showed resistance to periodate treatment. Additionally this epitope was not removed by phospholipase C treatment. Post-embedding immunogold labeling determined that 4F1

antibodies bound to the pellicle membrane on the surface of the parasite. Unfortunately, this reagent was mislabeled during laboratory purification, it was labeled as a monoclonal antibody and was later discovered to be from a mass culture. The cloning of the 4F1 hybridoma was not successful using the reagents available at this stage of the project, but 4F1 was subsequently cloned (Chapter 4).

Anti-*S. neurona* polyclonal antisera was produced in a rabbit. It reacts with antigens found in whole cell lysates, and it and immunoprecipitates a set of antigens similar to that set immunoprecipitated by CSF fluid from an EPM infected horse. The antibodies in hyperimmune rabbit serum recognize antigens on a Western blot in the size range of p29-30. These are antigens used to assist the diagnosis of EPM in horses as well as distinguishing infections caused by *S. neurona* or *Neospora*.[22, 51] When immunoblots of immunoprecipitated antigens were probed with 4F1 antibodies, the reactive antigen was not found in the Triton lysis buffer but was retained in the pelleted fraction (Figure 18, A and B). It appears that the immunoreactive antigen that is associated with the parasite membrane is also strongly anchored and co-precipitates with the insoluble parasite cytoskeleton. Control serum (data not shown) from a horse without antibodies to *S. neurona* did not precipitate either the 29 kDa protein or the cytoskeleton therefore we concluded there was an association with this antigen and the cytoskeleton. This cytoskeleton may be similar to the sub-pellicular microtubules of *T. gondii*.[77] Difficulty in solubilizing antigens in 1% Triton and the co-precipitation of proteins could cloud the interpretation of results seen in immunoblots of whole cell lysates of *S. neurona* that are cleared by centrifugation or immunoprecipitated using carbonate buffers containing 1% Triton X 100. [50]

We determined that a 29 kDa antigen of *S. neurona* was the immunodominant protein recognized on immunoblots by hyper immune rabbit antisera and immunoprecipitated by rabbit anti-*S. neurona* serum and equine CSF. It was possible to use immunoprecipitation by treating the preparation first with sonication followed by 2% Triton X-100 in the carbonate buffer. The data indicate that equine CSF did immunoprecipitate these same antigens, however the immunodominant antigen detected by CSF on an immunoblot is a 19 kDa antigen. It is possible that the rabbit recognizes linear epitopes on the 29 kDa protein thus indicating an immunodominant band at 29 kDa while the 19 kDa antigen recognized by the horse could be a conformational epitope. This observation has importance where diagnostic immunoblots are run under reducing conditions and reactions to conformational epitopes are not evaluated. Another interpretation of the data is that killed antigens stimulate a different immune response as opposed to live antigens. This is recognized in *T. gondii* and is a key factor in immunity as well as cyst formation in the intermediate host.[70,78] It may be possible that hyper immunization with killed organisms expressing the 29 kDa antigens may alter the response of the intermediate host when challenged with live *S. neurona*. If data presented by Liang and coworkers is correct and the antigens are important in invasion by the parasite and antibodies to this antigen are an important part of the horses immune response, the ultimate use of a vaccine derived from killed whole cells may not be effective because antibodies to this protein were not a dominant part of the immune response.[50] It is also possible that host species (rabbit vs. horse) may be responsible for the differences in antigens recognized. Liang and coworkers used clinical material that was derived from natural infections and our rabbit hyper immune serum was produced

using killed parasites.[50] Further studies need to be done to elucidate the relationship between the immune response to natural infections and those responses stimulated by killed organisms. Liang and coworkers also used 1% Trition X-100 to solubilize parasites for their studies and concluded that there was evidence that surface proteins Sn 14 and Sn 16 of *Sarcocystis neurona* merozoites are involved in infection and immunity.[50] They did not detect p29 on their immunoblots when Triton X-100 was used as shown in their Figures. They also concluded that this antigen was not recognized as specific protein for detection of *S. neurona* infection because a 30 kDa antigen immunoreactive with sera from horses with EPM is found in other *Sarcocystis* species. We determined that the 29 kDa antigen is not completely soluble in this detergent and may account for the result in which the 29 kDa antigen was not detected. Additionally, we propose that p29-30 as recognized on immunoblots is either two distinct proteins instead of just one, or two distinct pools of the same protein (perhaps one glycosylated and the other not). Certainly, the 29 kDa recognized in this study and the antigens reported by Liang are important in infection and immunity if neutralization assays, as proposed by Liang, do reveal significant differences in inhibitory activities between groups of serum and CSF samples with different immunoblot band patterns. Research will be required to elucidate the relationship of individual *S. neurona* proteins to the disease EPM. The cloning of genes that encode these proteins will facilitate their characterization and studies of their involvement in the immune response of the horse.

There are several technical difficulties that make monoclonal antibody production and hybridoma screening using *S. neurona* problematic. The first is that the parasites are intracellular parasites. We were able to purify merozoites away from host cells by using

calcium ionophore. We reported (in Chapter 2) that *S. neurona* merozoites changed phenotype in some host cell lines. We observed that switching the host cell used one passage prior to harvesting produced parasites that were noticeably larger and more homogenous in appearance. This apparent effect was interpreted as a synchronizing consequence on the parasite population with fewer schizonts remaining in the culture supernate, but rather, all parasites invading host cells. The events surrounding invasion that encourage all parasites to enter cells and begin forming immature schizonts could homogenize antigen expression. It was observed that amorphous free floating (undifferentiated) schizonts did not invade cells.

The second technical difficulty is hybridoma culture screening. The initial handling of the primary hybridomas routinely requires the use of equine serum. The survival of new hybridomas was compromised by 60 % when fetal calf or serum free media was used. It was possible to make the primary isolation in horse serum followed by weaning positive cultures into serum free medium. Additionally, we found that even though equine serum used in the isolation of monoclonal cultures was negative by both immunoblot and ELISA, this serum could competitively inhibit 4F1 antibodies binding to *S neurona* proteins when solubilized proteins were used in a competitive ELISA. We did not determine the method of competitive inhibition of normal serum with our monoclonal antibody. It is possible that *S. neurona* exhibits non specific binding as has been shown for *T. gondii*.[73]

The antibody preparations we employed were directed against the immunodominant antigens of *S. neurona*. Equine serum, CSF from histopathologically confirmed cases of EPM, and hyperimmune rabbit anti-*S. neurona* serum all recognize a

29 kDa protein when they are used to probe immunoblotted proteins of *S. neurona*. Other apicomplexan parasites: *T. gondii*, *S. muris*, and *Neospora* all have species specific surface proteins at approximately 30 kDa. By characterization of the major surface antigens of *S. neurona* the predominant antigens recognized by antibodies stimulated in the humoral immune response of the horse can be examined. It has been shown in *T. gondii* that the major surface antigen, SAG1, is a member of a protein family whose members contain glycolipid anchors. In addition, surface antigens of *Neospora* have some similarity to SAG1indicating that there is importance to structure or function of these surface proteins that has been conserved with evolution. It is likely that the 29 kDa protein recognized by 4F1 belongs to the family of antigens shared by other apicomplexan parasites, however the epitope to which 4F1 reacts is specific for *S. neurona*. It is important to examine the different stages of *S. neurona* to determine if the expression of p29 changes. Certainly *T. gondii* has been shown to express bradyzoite specific proteins and it is probable *S. neurona* does also.

We determined 4F1 antibodies recognize two spots when used to probe two dimensional blotted antigens of *S. neurona*. Preliminary data obtained with trypsin treated parasites raise the question that 4F1 recognize either two distinct proteins instead of just one, or two distinct pools of the same protein (perhaps one post translationally modified and the other not). The answer to these questions can be elucidated by epitope mapping of the cloned SnMSA-1.

Questions remain concerning the interpretation of the 19 kDa protein reported by several groups as important in infection, however answers await the cloning and characterization of this antigen.[22,50-51] It is possible that the p29 examined in this study

can be degraded and detected by immunoblot as an approximate p19. Further studies need to be done to confirm this observation. Detection of *S. neurona* infection by demonstration of reactivity of serum and CSF samples with the Sn 11, Sn 14, and Sn 16 antigens has been extensively used as a diagnostic tool. In contrast to Liang, we found that the 29 kDa antigen was the immunodominant antigen precipitated by CSF from a horse with EPM (and from which *S. neurona* was cultured).[50] Immunoprecipitation is a powerful tool to concentrate and purify antigens although it is possible that co-precipitation of non-antigenic proteins may complicate the interpretation of data. Recombinant antigens for the immunodiagnosis of bovine *N. caninum* rely on 33, 36, 28-34 kDa proteins.[20] Marsh reported *Neosporosis* as cause of equine protozoal myeloencephalitis and found the CSF from the affected horse reacted to a number of *S. neurona* proteins; however, only 2 immunodominant bands of approximately12 and 29 kDa had the same molecular weight as those recognized in the *S. neurona*-infected horses.[22] The monoclonal antibody that reacted with p29 in this study did not react with *Neospora* by ELISA, IFA (data not shown) and Western blot (data not shown). The use of p29 of *S. neurona* as a diagnostic antigen may be able to differentiate *S. neurona* or *Neospora* as the etiologic agent in EPM in the horse using 4F1 monoclonal antibodies in a competitive ELISA.

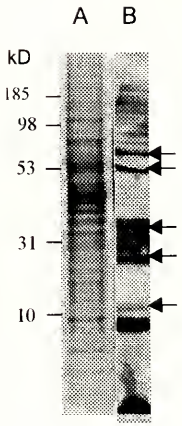


Figure 5. Comparison of SDS-PAGE and Western blot analysis of *S. neurona* merozoite proteins. Lane A, total *S. neurona* proteins stained with coomassie blue; Lane B, *S. neurona* antigens recognized by polyclonal anti-*S. neurona* antibodies are marked by arrows.

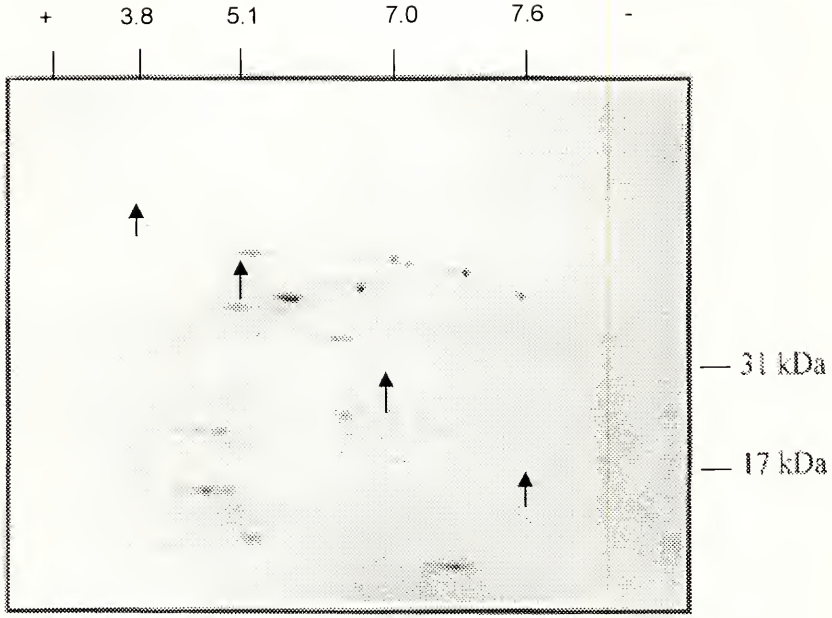


Figure 6. 2D electrophoresis of *S. neurona* proteins stained with coomassie blue stain. Arrowheads mark approximate pI when compared to standards. The standards are from left to right: amylogulcosidase, 89 and 70 kDa, pI 3.8; ovalbumin 45 kDa, pI 5.1; carbonic anhydrase 29 kDa, pI 7; myoglobin 17 kDa, pI 7.6.

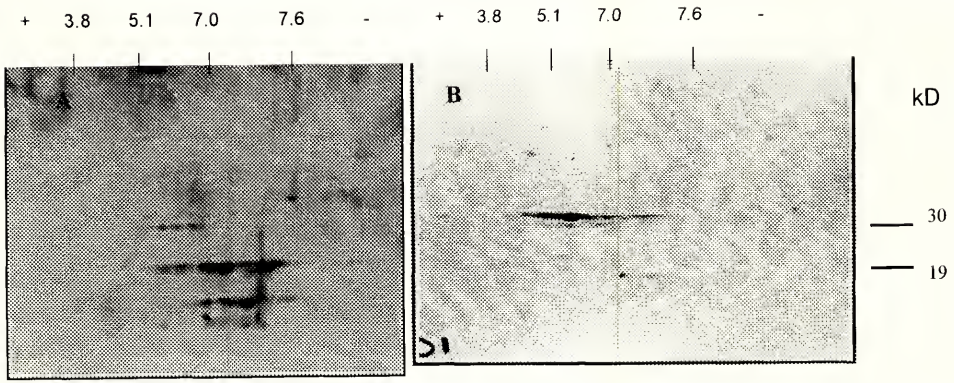


Figure 7. Western blot analysis of total *S. neurona* proteins separated by 2D gel electrophorsis and probed with CSF from a histopathologically confirmed case of EPM, Panel A; and with antibodies from a hyper-immunized rabbit, Panel B.

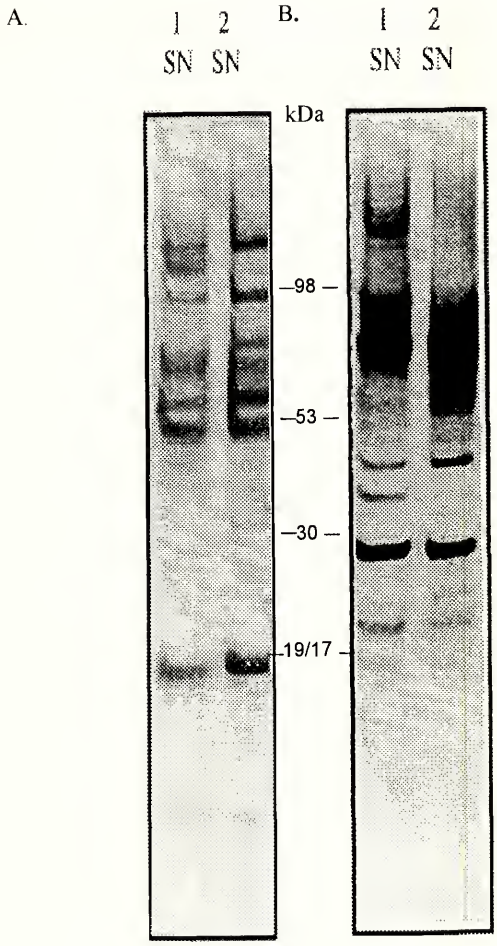
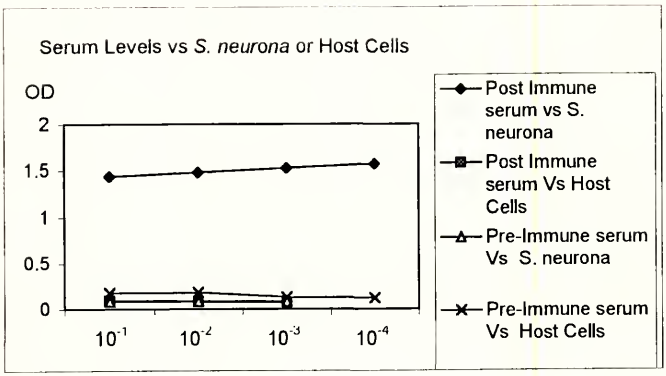


Figure 8. Western blot analysis of 1% Triton X-100 insoluble *S. neurona* antigens. Panel A, Detected with CSF fluid from a horse with histopathologically confirmed EPM; panel B, detected with polyclonal anti-*S. neurona* antisera. Lane 1, antigens immunoprecipitated with equine CSF; lane 2, antigens immunoprecipitated with polyclonal anti-*S. neurona* serum. Position of molecular size standards are marked kDa.



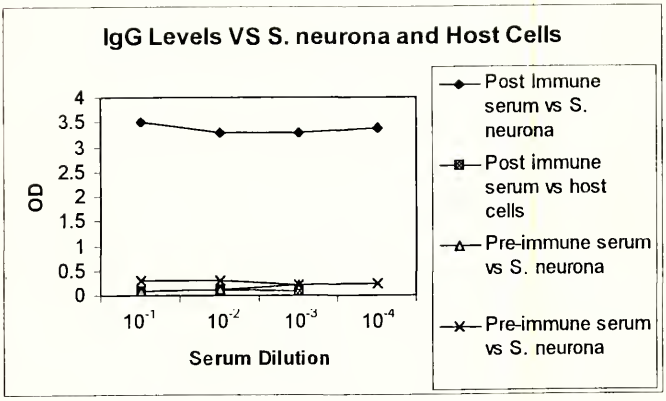


Figure 9. ELISA analysis of a mouse immunized with solubilized antigens of *S. neurona* merozoites. Whole serum levels show a good response to *S. neurona* but not host cells.

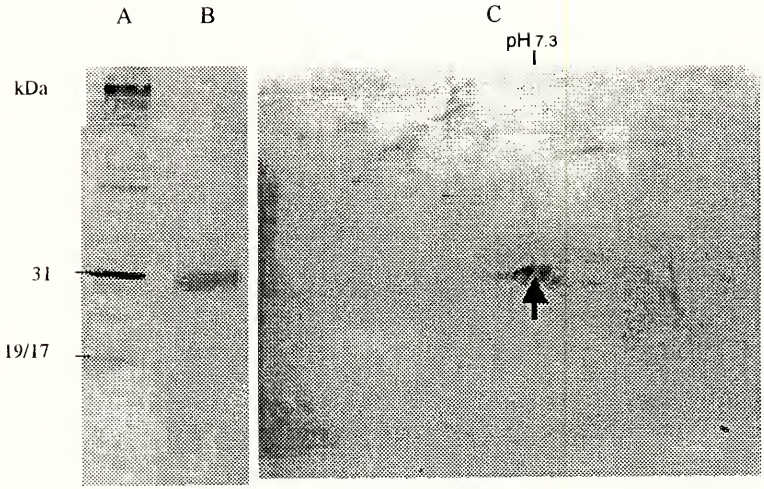
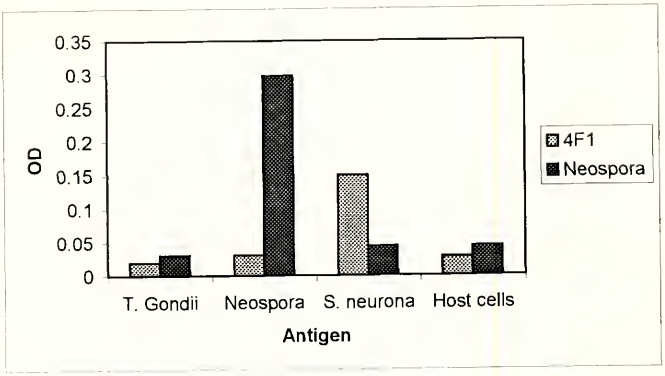


Figure 10. Western blot analysis of *S. neurona* merozoite proteins. Panel A, blot of SDS PAGE gel probed with hyper immune mouse serum prior to fusion. Panel B, blot of SDS PAGE gel probed with hybridoma mass culture supernatant from which MAb 4F1 was derived. Panel C, blot of *S. neurona* merozoite proteins separated by 2D gel electrophoresis probed with the Mab 4F1. Arrow points to a 29 kDa protein that migrated as 2 spots at pI 7.3.

A.



B.

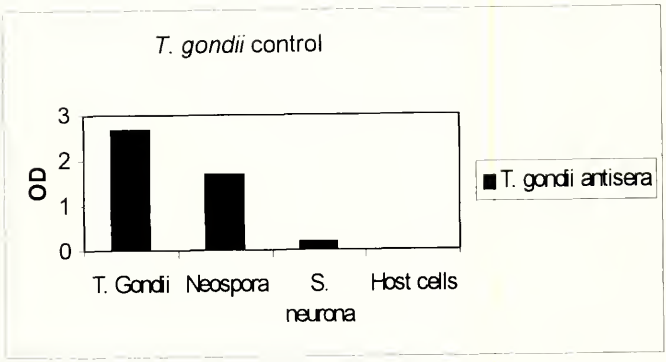


Figure 11. ELISA analysis to determine the cross-reactivity of 4F1 monoclonal antibody to other apicomplexa known to infect horses. The graph of the data is shown in A, indicating that 4F1 does not bind *Neospora*, host cells, or *Toxoplasma*. In this assay the *T. gondii* antiserum was not specific and the assay was repeated, B.

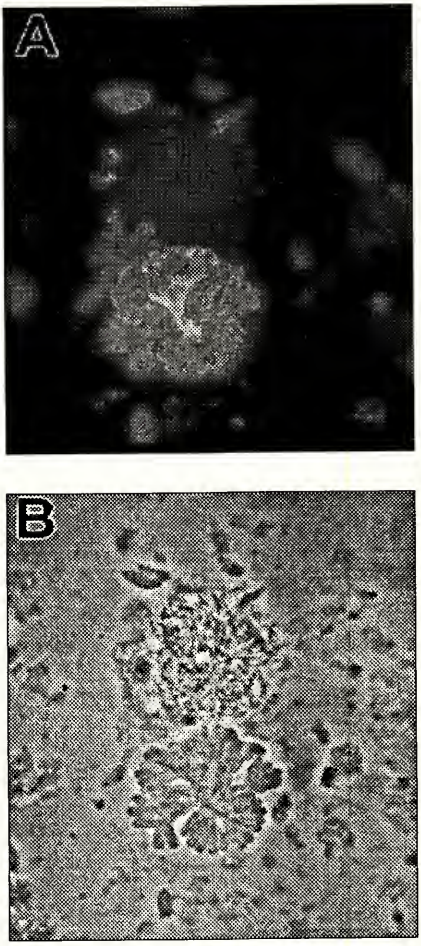


Figure 12. Indirect fluorescent antibody assay showing the binding of 4F1 to the surface of formalin fixed parasites. Panel A shows formalin fixed parasites treated with 4F1 antibodies. Panel B shows the phase contrast image of the same field. The controls for this experiment, pre-immune mouse serum and isotype control also showed no binding. Control data is represented in the figures demonstrating surface binding by post-embedding, immuno-gold labeling, Figure 13, and ELISA, Figure 9.

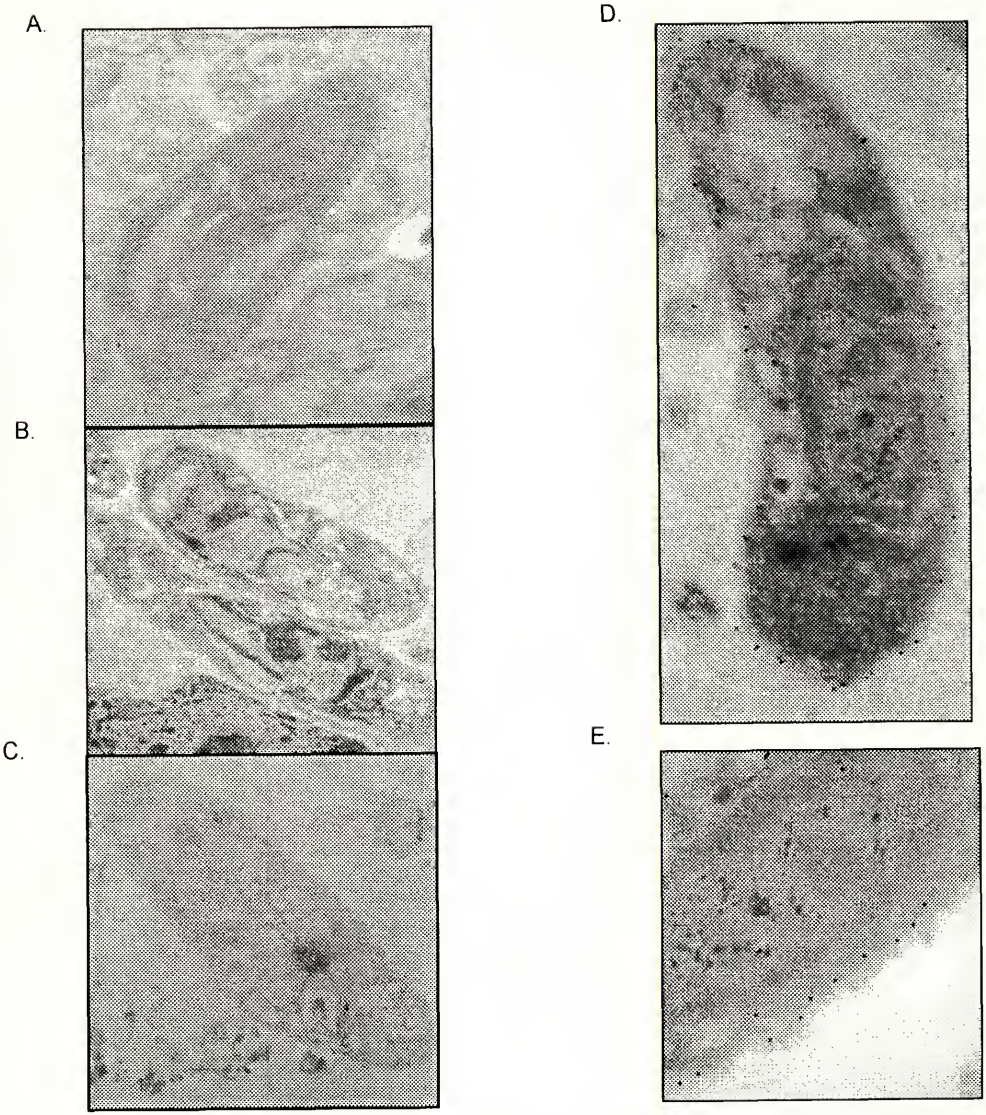


Figure 13. Immunogold labeling of *in vitro S. neurona* merozoites using 4F1 mass culture antibody shows that the surface of the parasite is labeled, Panel A. Controls included a mouse monoclonal isotype IgG1, Panel B. Phospholipase C treated parasites, Panel C, were labeled as in Panel A. Cloned hybridoma 1631 monoclonal antibodies derived from 4F1 are shown binding to the surface of *S. neurona*, Panel D. Panel E shows the detail of the immunogold labeling in Panel D.

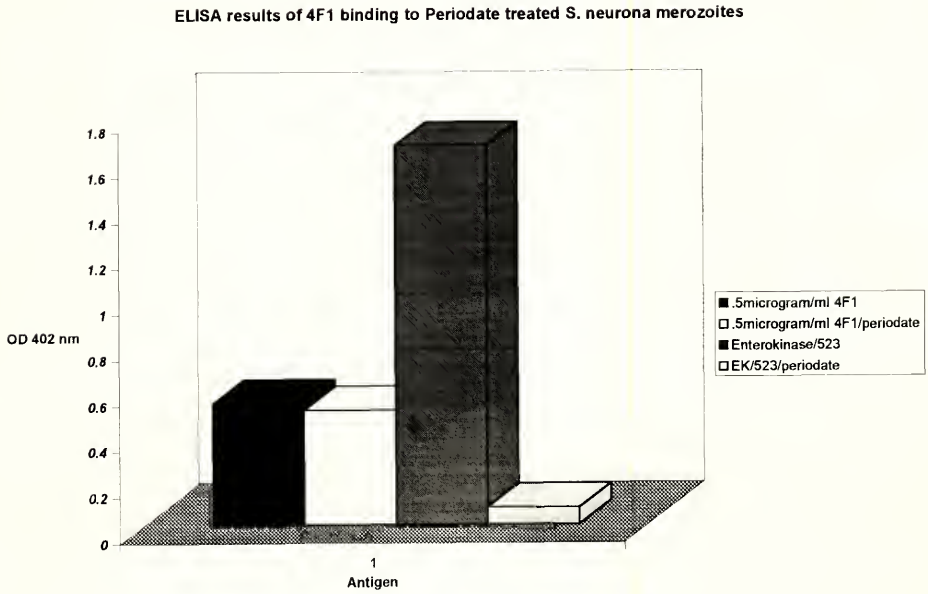


Figure 14. Bar chart of ELISA analysis to determine the nature of the epitope detected by 4F1 mass culture antibodies. Periodate treatment did not remove the epitope recognized by 4F1 while the enterokinase control was detected by monoclonal antibody to enterokinase and this epitope was removed with periodate treatment. Results consistent with those shown here were obtained by immunoblot and post-embedding immunogold labeling electron microscopy (data not shown).

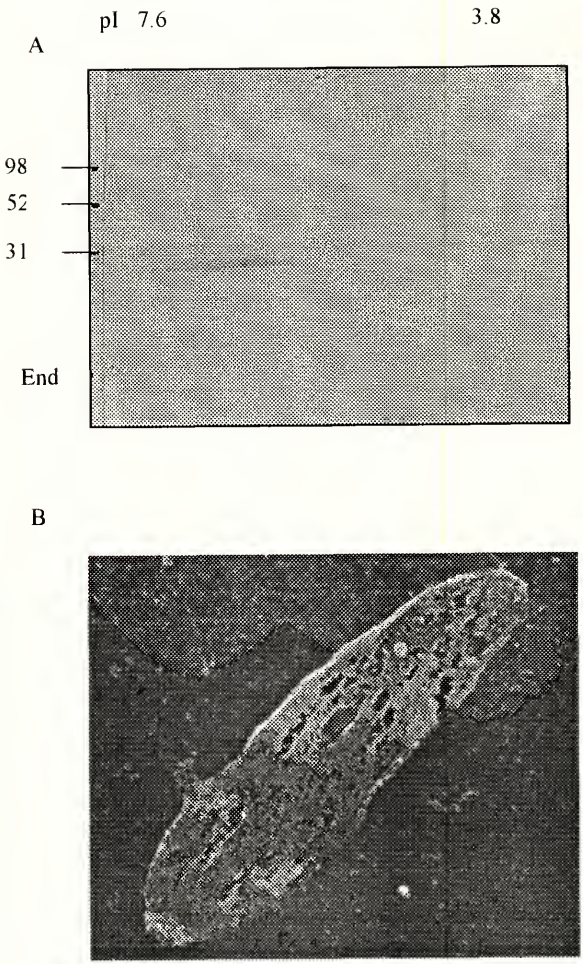


Figure 15. Western blot analysis of *S. neurona* 1% Triton X-100 insoluble protein fraction separated by 2D gel electrophoresis. Panel A was probed with 4F1 antibodies; panel B is the material contained in the insoluble fraction shown by electron microscopy.

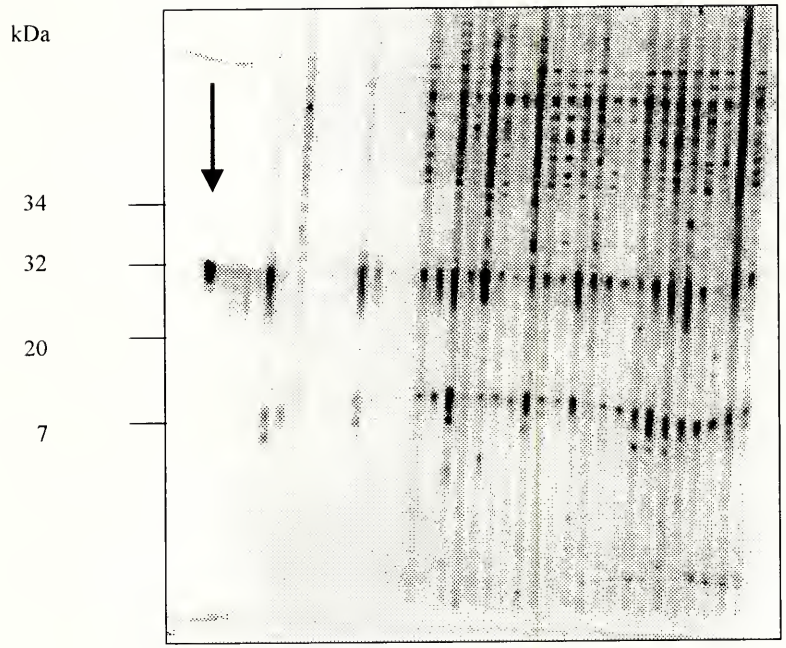


Figure16. Western blot analysis of proteins from the Sn3 isolate of *S. neurona*. The arrow marks the lane probed with 4F1 detecting a 28 kDa antigen. The other lanes on this blot are lanes probed with equine serum or CSF samples, shown is a 28 kDa protein detected by these clinical samples.

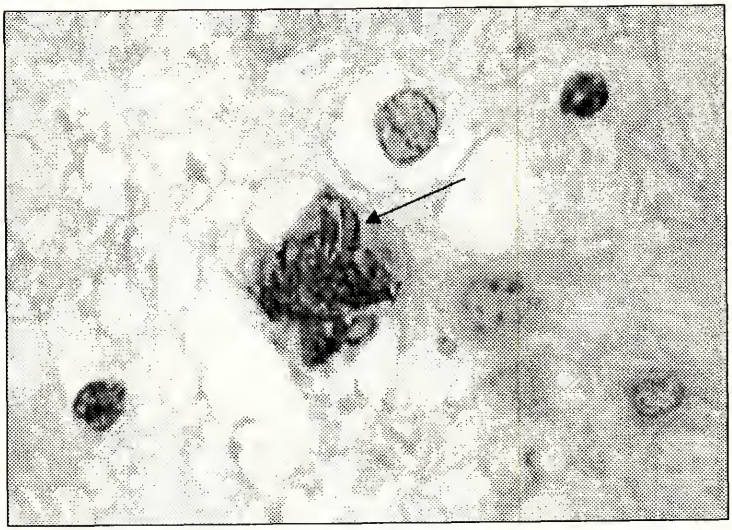
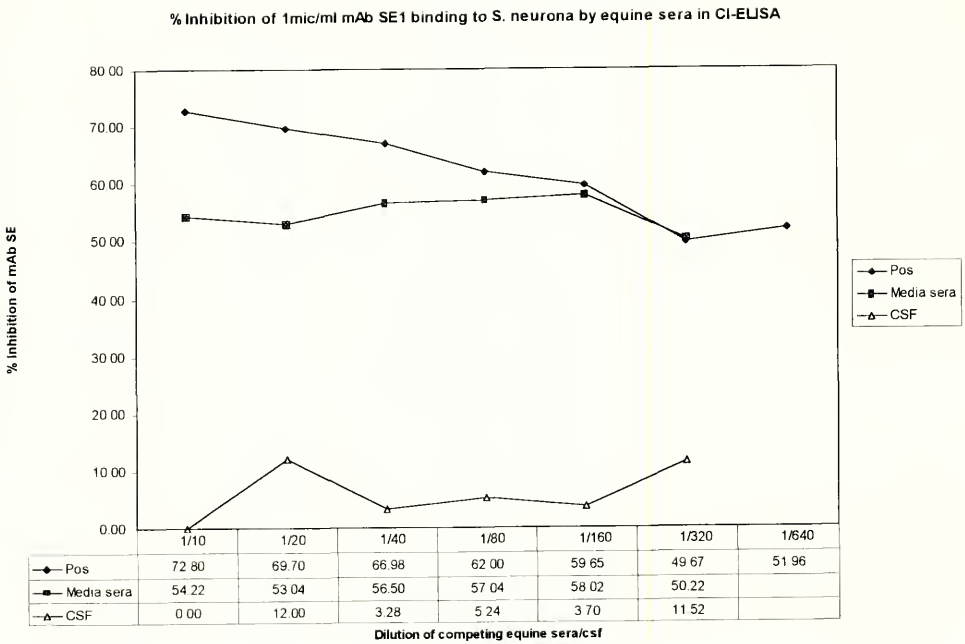


Figure 17. Tissue section from the spinal cord of a horse with clinical signs of EPM. Arrow indicates positive reaction when 4F1 monoclonal antibody was used in an immuno-histochemical assay to detect *S. neurona*.



	1/10	1/20	1/40	1/80	1/160	1/320	1/640
Pos	72.80	69.70	66.98	62.00	59.65	49.67	51.96
Media sera	54.22	53.04	56.50	57.04	58.02	50.22	
CSF	0.00	12.00	3.28	5.24	3.70	11.52	

Figure 18. Competitive ELISA that measured the percent of inhibition of 1 microgram/ml of 4F1 by equine serum and CSF.

CHAPTER 4
CLONING AND EXPRESSION IN *ESCHERICHIA COLI* OF CDNAS ENCODING SURFACE ANTIGENS OF *SARCOCYSTIS NEURONA*

Introduction

Equine protozoal myeloencephalitis is an important neurological disease of horses caused primarily by *Sarcocystis neurona*.[6] *Sarcocystis neurona* has been cultured from CNS lesions of horses from several different locations in the United States and reported to show only minor differences when compared by morphologic, immunologic, and DNA tests.[3,19,29] Other species of *Sarcocystis* have been described as disseminating in the intermediate host via the blood stream.[40-42] Recently, a foal with a compromised immune system (SCID) was infected with *S. neurona* sporocysts and *S. neurona* merozoites were isolated from the circulatory system. At some point during the infection in the horse, *S. neurona* travels to the brain and spinal cord, where merozoite stages of this parasite replicate and cause pathology. During the infection the merozoite stage is exposed to the host's immune system, thus it is possible that prior exposure to merozoite surface antigens under the right conditions may elicit a protective immune response. Cloning and expressing genes, which encode merozoite surface antigens, are important steps in preparing specific immunologic reagents useful in studies of *S. neurona* infection in the horse.

Cultured merozoites of *S. neurona* are used as the antigens in the immunoblot testing of horses to demonstrate the presence of antibodies produced by infected horses.[12] Native antigens recognized by equine infection sera in these assays may be considered as candidates for further characterization. Since *in vitro* cultured merozoites can be prepared so as to minimize host cell contamination, they furnish a source of polyadenylated mRNA to construct a cDNA library representing the information expressed by the genome of *S. neurona* during the merozoite stage.[55] We report the construction of a cDNA library and the subsequent identification, sequencing, and expression of a gene encoding a 29 kDa protein found on the surface of the merozoite stage of *S. neurona*. The protein encoded by this gene, termed SnMSA1, is an immunodominant antigen recognized on protein blots. The original clone was identified in a collection of random sequence tags prepared to characterize our cDNA library. Additional clones of the gene were obtained to identify a full-length copy. The nucleotide sequence of a gene encoding the complete coding region was determined, and the gene was sub-cloned into an expression vector. The recombinant protein (rSnMSA1) was expressed and characterized.

Materials and Methods

Growth and Preparation of *S. neurona*

Sarcocystis neurona merozoites were cultured as previously described.[55] Briefly, the UCD1 isolate was obtained from the spinal cord from a horse diagnosed with EPM at the University of California and was the generous gift of Dr. Antoinette Marsh. This isolate has been maintained in bovine monocyte cells cultured in RPMI media

supplemented with 10% bovine serum at 37 C in a 5% CO_2/air atmosphere. Established cell lines and primary cultures were maintained in plastic culture flasks incubated under the same conditions and containing media plus 10 % fetal calf serum and 1X pen/strep (Gibco). Host cells were released from the culture flask surface by trypsin treatment and transferred to fresh culture flasks at a density of $2X10^4$ per cm^2 resulting in a monolayer that was ~60% confluent. Parasites were immediately added at a density of $2X10^3$ per cm^2. After 3 days of incubation, growth was monitored by counting infective foci under the microscope. Cell monolayer was washed daily with 15 ml media plus supplements to remove any free cells. Parasites were routinely harvested using a 1μM calcium ionophore solution when their density approached an average of 5-10 parasites developing within the cells visible in a single microscopic field (400x). Merozoites at twelve days post infection were released with calcium ionophore and harvested in Hanks buffered salt solution. The free parasites in the culture medium were monitored by cytospin followed by Geimsa staining. Washed merozoites were added to RNAzol as per manufacturer recommendations. The total RNA was separated on a denaturing formamide gel to evaluate the amount of host cell contamination.[78] The selection of polyadenylated RNA (poly A-RNA) from total RNA was achieved by the use of oligo dT magnetic beads (Promega) as per manufacturer recommendations.

Library Construction

Two methods were used to construct cDNA expression libraries. The method of Froussard was used to produce a cDNA library in the lambda vector Uni Zap XR.[79] First strand synthesis was prepared using a 26 nucleotide primer containing a random

hexameter at the 3' end (universal primer $-dN_6$; 5'-GCCGGAGCTCTGCAGAATTCNNNNN-3'). *S. neurona* poly-A RNA, 0.35µg, was suspended in 6µl of distilled water, heated to 65 C for 5 min, rapidly cooled on ice and reverse transcribed after addition of 0.5 µl (20 U) RNAsin, 1.25 µl 10 X reverse transcription buffer (500 mM Tris-HCl, pH 8 at 43 C, 800 mM NaCl, 80 mM $MgCl_2$, 50mM DTT), 1.25 µl dNTP (10mM), 1.5µl universal primer $-dN_6$ (0.1µg/µl), 2 µl (16 U) AMU reverse transcriptase. Incubation was at 43 C for 1 hour. The reaction was then boiled for 2 min. and rapidly cooled on ice. For second strand cDNA synthesis the following components were added: 24.25 µl dH_2O, 10 µl 5 X Klenow buffer, 1.25 µl 5 methyl dCTP 100 mM), and 2 µl Klenow fragment (8 U). After 30 min. incubation at 37 C the sample was purified on a chromaspin – 400 column (Clontech) to remove the universal primer d-N_6. One microliter of the randomly synthesized double stranded cDNA population was amplified in the presence of 1mM universal primer in a 50 µl reaction mix containing 50mM KCl, 1.5 mM $MgCl_2$, .01% gelatin, 500mM each dNTP and 1.5 U TAQ polymerase. The samples were subjected to 40 cycles of amplification: 94 1 min., 55 C 1 min., and 72 C 3 min. Final amplification products were analyzed on a 0.8% agarose gel. The amplified cDNA fragments were ligated to Eco R1 adapters and separated on a drip column containing Sepharose CL-2B gel filtration medium. The size fractionated cDNA was precipitated and ligated to the Uni Zap XR vector. The lambda library was packaged using a high efficiency Gigapack III gold packaging extract and plated on the *E. coli* cell line XL1-Blue MRF^-.

A second cDNA expression library was constructed using a lambda ZAP vector. Poly A-RNA prepared as above was converted into double stranded cDNA using a

Stratagene λ Zap cDNA synthesis kit as per manufacturer recommendations. After *in vitro* packaging and transfection of host cells (XL1-Blue MRF') the primary library was amplified to yield a high titer phage stock. The sequencing of random clones was accomplished after *in vivo* excision of one ml of the amplified library. The excised phagmids were transfected into SOLR cells following manufactures recommendations.

Library Screening

Polyclonal anti-*S. neurona* rabbit antibodies were produced by Lampire Laboratories with whole parasite preparations that had been fresh frozen and shipped. Antibody titer production was monitored by both immunoblotting and ELISA. Screening according to standard procedures. Primary antibodies were diluted 1:500. Alkaline phosphatase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG was used as secondary antibodies (dilution 1:3000). Positively reacting phage plaques were isolated and subjected to 3 additional rounds of screening. Positive phagmid clones were converted into pBluescript SK (-) plasmids by means of *in vivo* subcloning.

The *in vivo* excision protocol (Stratagene) was followed. Briefly, 1 µl of the amplified library was diluted by addition of SM buffer to give 400 pfu/µl. Overnight cultures of XL1-Blue MRF' and SOLR, supplemented with 0.2% (w/v) maltose and 10mM $MgSO_4$ were centrifuged and the media removed. The cells were resuspended to an OD_{600} of 1 in 10 mM $MgSO_4$. Two hundred microliters of XL1-Blue MRF' were added to 250 µl ($1X10^5$) phage and 1 µl Ex Assist helper phage ($1 X 10^6$ pfu/µl) and incubated at 37 C 15 min. in a Falcon 2059 polypropylene tube. Three ml of LB broth was then added and the tube incubated 2 hours at 37 C with shaking. The tube was heated to 70 C for 20 min. and centrifuged at 1000 g for 15 min. The supernate

containing the excised pBluescript phagmid was plated by adding 200 µl SOLR cells to 100 µl phage supernate and incubated for 15 min at 37 C. Two hundred µl of the cell mixture was plated on LB-ampicillin agar plates (50µg/ml) and incubated overnight.

Additionally, 96 random clones were selected, grown overnight, and plasmids isolated by standard mini-prep procedures. The isolated plasmids were partially sequenced using the T3 primer, and one candidate clone, C10, was selected based on BLAST analysis.[80] The cDNA library was screened by hybridization with a digoxigenin-labeled probe (Eco R1/Xho1), the fragment from clone C10.

The clone was prepared for subsequent expression in pET14b via PCR. PCR primers 5' GGGGGGATCCGATGACGAGGGCGGGTGCTGCTG and 3' GGGGGATCCTTAGCAAAAGTGCAAGAAAGCG were designed by adding Bam H1 nucleotide sequence to the sequence encoding the 5' and 3' ends of the open reading frame of the full SnMSA1 gene. A PCR reaction was performed in buffer containing 49µl PCR reaction buffer, [5µl 10X buffer, 3µl dNTP, 1µl forward primer, 1µl reverse primer, 38µl dH_2O, 1µl TAQ polymerase] with the PCR conditions 93 C for five min. with hot start, 2 cycles: 93 C 1 min, 65 C 1.5 min, 72 C 1.5 min, followed by 30 cycles 93 5 sec, 65 30 sec, 72 1 min followed by 10 min. at 72 C and held overnight at 4C, using 1µl second strand synthesis reaction as template. The single amplification product produced by PCR was isolated by electrophoresis and cut out of a low melting agarose gel followed by TOPO (Invitrogen) cloning. One colony was selected. The size of the insert was determined by electrophoresis and the sequence was confirmed by sequence analysis. The DNA was then cloned into the BamH1 site of pBluescript, transfected into XL1-Blue

MRF, this allowed the color selection of clones containing the insert. The DNA insert was then subcloned into the expression vector, pET 14b, for protein expression.

Expression of Fusion Protein

Uncloned *S. neurona* cDNA was used as the template for PCR amplification of the coding region by PCR using oligonucleotides GGGGGATCCGATGACGAGGGCGGGTGCTGCTG and GGGGGATCCTTAGCAAAAGTGCAAGAAAGCG as forward and reverse primers, respectively. Primers were designed by adding the Bam H1 recognition sequence to the sequence encoding the 5' and 3' ends of the open reading frame of the full SnMSA1 coding region. Amplification was carried out using the TaKara kit following manufacturer's recommendations (TaKara, Fisher Scientific, Pittsburgh, Pa.) in a reaction cycle of 93 C,5 sec, 65 C, 30 sec, 72, 1 min repeated 30 cycles. The single amplification product was isolated by agarose gel electrophoresis and cloned into the TOPO cloning vector (Invitrogen, Carlsbad, CA.). The sequence of the TOPO clone was determined. The full-length open reading frame of SnMSA-1 was subcloned from the TOPO clone into the BamH1 site of the expression vector pET14b. Clones in the correct orientation were identified by restriction mapping and selected clones were sequenced.

The clone in pET 14b was prepared for subsequent expression in *E. coli* BL21 cells. Bacteria carrying the recombinant plasmid were grown at 37C to an A_{600} of 0.6 in a 50 ml culture. Twenty ml of culture was added per 1 liter of LB-ampicillin (50 μg/ml) broth and grown at 37 C to an A_{600} of 0.6. Isopropyl thioβ-d-galactoside (IPTG) was then added to a final concentration of 0.4 mM and incubation was continued for 2 hours.

After harvesting the cells by centrifugation, the fusion protein was detected in whole cell extracts on immunoblots using an anti-His-tag monoclonal antibody.

Preparation of rSnMSA1 Protein

Four liters of transformed BL21cell culture grown and induced with IPTG as described above were collected by centrifugation at 3000 x g at 4C for 20 min. Cells were washed once with PBS and inclusion bodies purified as described by the manufacturer (Novagen, Madison, Wisconsin) except the cells were disrupted by passing them through a French press. Inclusion bodies were solubilized in TN buffer (50mM Tris, pH 7.4, 150 mM NaCl, 1mM $MgCl_2$) containing 6M urea for 40 min. at 4C. Insoluble material was removed by centrifugation at 8700 x g for 30 min, at 5C. A His-Bind column (Novagen) was prepared by washing 1 ml resin with three volumes distilled water followed by five volumes TN buffer containing 6M urea. The solubilized inclusion bodies were added to the column, and it was washed with 10 volumes TN buffer containing 6 M urea, pH 6. The recombinant protein was eluted using TN buffer containing 6M urea, pH 4.5, and subsequently dialyzed to change the buffer to 10X thrombin cleavage buffer (200 mM Tris-HCl, pH 8.4, 1.5 M NaCl, 25 mM $CaCl_2$ 2M urea, and 1% Triton X 100) using a spin separator (Micron). Thrombin cleavage was performed using the manufacturer's recommendations (Novagen, Madison, Wisconsin).

Production of Polyclonal Anti-sera in Rabbits and Mice

Polyclonal anti-*S. neurona* rabbit antisera was prepared at a commercial laboratory (Lampire) following 3 immunizations using Ribi adjuvant. The anti-rSnMSA-1 polyclonal mouse anti-sera was prepared by the University of Florida Hybridoma Core

facility. The monoclonal antibody, 2A7, was the gift of Antoinette Marsh and found to bind to the surface of *S. neurona* by IFA as well as a 29 kDa antigen on immunoblots of whole *S. neurona* merozoite proteins. The 2A7 monoclonal antibody was used to perform IFA, immunogold-label electron microscopy, and to develop immunoblots. The monoclonal antibody, 2A7, was prepared at the University of Missouri. It binds to the surface of *S. neurona* merozoites as detected by IFA, and it binds to a 29 kDa protein on immunoblots of electrophoretically separated whole merozoite extracts. Serum from European horses was used as a negative control and CSF from a horse with histologically confirmed *S. neurona* encephalomyelitis was used as a positive control.

Proteolytic removal of the His Tag from the Fusion Protein

Thrombin cleavage was achieved using the manufacture recommendations. Briefly, optimal conditions of reaction were found by performing small scale digestions and the extent of cleavage determined by SDS-PAGE analysis. The thrombin was diluted 1:25, 1:50, 1:100, and 1:200 to contain 0.04, 0.02,0 .01, and 0.005 U enzyme per µl. The reaction components were assembled in a labeled 1.5 ml microfuge tube: 10µl thrombin cleavage buffer containing 12 µg target protein was added to each tube containing 1µl enzyme dilution. The negative control was 1µl storage buffer without enzyme. Thirty-nine µl deionized water was added to a final volume of 50µl. Ten µl aliquots were removed into 2X LDS sample buffer after 2, 4, 8, and 16 hours. The extent of cleavage was followed by SDS-PAGE and immunoblots using the anti-His-tag monoclonal antibody. Cleavage was complete after 8 hours incubation at room temperature in 1U/ul of thrombin. The reaction was scaled up to permit cleavage of 3 mg protein. After cleavage was complete the biotinylated thrombin was removed with

streptavidin agarose using a ratio of 16 µl settled resin (32 µl of the 50% slurry) per unit enzyme. The buffer was changed using a spin filter and the appropriate buffer. For IEF the protein was placed in 125 ul sample buffer (6M urea, 1% CHAPS, 15mM DTT, 0.2% Bio-Lytes [pH 3-10]), and the IPG strip (Bio-Rad) was rehydrated with the protein solution. Isoelectric focusing was performed at 20,000 V hours. Following isoelectric focusing the strip was equilibrated in two steps. The first step equilibration buffer (6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20 % glycerol, 130 mM DTT) was used for ten min. followed by the second step equilibration buffer (6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20 % glycerol, 135 mM Iodoacetamide) for ten min.. The proteins were separated in the 2nd dimension using a ZOOM (Invitrogen) 4-12% Bis-Tris gel by electrophoresis at 200 v for 30 min. The gels were stained with colloidal blue or blotted to PVDF membranes for immunoblot assay.

Southern and Northern Blot Hybridizations

Sarcocystis neurona merozoites were harvested from cultures at 12 days post infection, and the total DNA was extracted using standard procedures. Briefly, the whole cells were pelleted in PBS. The pellet was suspended in lysis buffer (1M Tris, pH 8, 0.5 M EDTA, pH 8, 5 M LiCl, 1%Triton X 100). An equal volume of phenol: chloroform: isoamyl alcohol (1:1:25) was added and mixed by inversion for 2 min.. The suspension was centrifuged at 10,000 rpm in a microfuge at 4 C for five min. to separate the liquid phases. The aqueous phase was removed to a new tube and 2.5 volumes of 100% ethanol was added and incubated for 20 min at –20. The precipitated DNA was pelleted by centrifugation at 14,000 rpm for 20 min at 4 C. The pellet was washed in 80% ethanol, air dried and resuspended in 50 µl TE. DNA was extracted from *Toxoplasma gondii,*

Neospora caninum, and *Sarcocystis falcatula* in a similar fashion. DNA (5µg) aliquots were digested with EcoR1 or BamH1 and electrophoresed on a 0.8% (w/v) agarose gel. Southern blotting was performed by capillary transfer onto nylon membranes following acid depurination, alkaline denaturing, and neutralization steps.

Nucleic Acid Blotting and Analysis

Sarcocystis neurona merozoites were harvested from cultures at 12 days post infection, and the total DNA was extracted using LiCl and phenol/chloroform. (Protocols and applications guide, Promega) Briefly, partially purified merozoites in PBS were pelleted by centrifugation. The pellet was suspended in lysis buffer (1M Tris, pH 8, 0.5 M EDTA, pH 8, 5 M LiCl, 1%Triton X 100) followed by phenol chloroform extraction and ethanol precipitation. The precipitated DNA was pelleted by centrifugation ,washed in 80% ethanol, air dried and resuspended in 50 µl TE. DNA was extracted from *Toxoplasma gondii*, *Neospora caninum*, and *Sarcocystis falcatula* in a similar fashion. DNA (5µg) aliquots were digested with EcoR1 and electrophoresed on a 0.8% (w/v) agarose gel. Southern blotting was performed by capillary transfer onto nylon membranes following acid depurination, alkaline denaturing, and neutralization steps.

For Northern analysis, total RNA was size fractionated by agarose/formaldehyde gel electrophoresis and transferred to nylon membranes. (Promega) After pre-hybridization (2XSSC containing salmon sperm DNA)for two hours at 60 C, a probe prepared from the open reading frame of SnMSA-1 was ^{32}P labeled by the random primer method and added to the hybridization buffer for 24 hours at 60 C. The membrane was washed in 0.2XSSC, 0.2% SDS at 55 C. Autoradiography was performed with enhancing screens for 8 days at -80 C.

Southern blotting and hybridizations were performed following standard procedures. (Protocols 1996). The labeled probe was used to hybridize blots of DNA and mRNA from *S. neurona*, *T. gondii*, *N. caninum*, and *S. falcatula* and the bands were visualized by autoradiography as described above.

Immunoblot Analysis

Protein samples were suspended in 4X SDS-PAGE gel loading buffer (1.09 M glycerol, 141 mM Tris base, 106 mM Tris HCL, 73 mM lithium dodecyl sulfate, 0.51 mM EDTA, 0.22mM Serva Blue G250, 0.175 mM phenol red, pH 8.5). After boiling samples were loaded onto 4-12% New Page Bis Tris gels (Invitrogen). For Western blot analysis proteins were transferred onto PVDF membranes and immunodetection was as previously described.

Production of Polyclonal Anti-sera in Mice

Mouse monoclonal and polyclonal anti-sera were prepared by the University of Florida Hybridoma Core facility as described in chapter 3. The humoral response to the recombinant protein was monitored by ELISA using serial dilution of whole serum and immunoblot using serum diluted 1:1000.

Capture ELISA was Used to Quantitate S. neurona Antigens

Antibodies raised against *S. neurona* were used to quantitate rSnMSA-1 that bound in a capture ELISA. The capture ELISA using rSnMSA-1 served as a standard to measure the amount of SnMSA-1 used in preparative samples for immunoblot. First, recombinant protein was used in an ELISA to titer purified IgG from polyclonal rabbit anti-*S. neurona* antibodies. A second aliquot of purified antibody was biotinylated and

used as a reagent to detect antigen captured by antibodies, this reagent was also quantitated using against rSnMSA-1. Both protein G purified and biotinylated protein G purified rabbit anti-*S. neurona* were used to configure a capture ELISA. 96 well plates were coated with protein G purified rabbit anti-*S. neurona*, followed by the addition of diluted rSnMSA-1. After incubation and washing using standard conditions biotinylated protein G purified rabbit anti-*S. neurona* was added, incubated, and washed. The detection of the biotinylated antibodies was accomplished with the addition of alkaline phosphatase-Strepavadin (P-NPP,(Sigma N2765) and the absorbance read at 405 nm. Additionally, the purified rabbit anti-*S. neurona* was used as the capture antibody and monoclonal antibodies directed against SnMSA-1 were used for detection of protein captured.

DNA Sequencing

DNA sequencing was performed by the dideoxy dye terminator method (Perkin Elmer) at the ICBR Molecular Services Core facility at the University of Florida. Sequence analysis programs used were GCG, (Madison, WI) ClusterL, and Jellyfish (Biowire.com).

Protein Analysis

The mw of the recombinant protein was verified by mass spec completed at the University of Florida Protein core.

Results

Monoclonal antibody 1631 binds a 29 kDa antigen on immunoblots of SDS-PAGE separated native antigens derived from cultured *S. neurona* merozoites and the

surface of *S. neurona* merozoites by post embedding immunogold labeling (Figure 19). Also, monoclonal antibodies 1631 and 2A7 strongly react with the surface of fixed or unfixed merozoites by IFA (Figure 19). When the antigen preparation is reduced by heating in the presence of β-mercaptoethanol, antibody binding is greatly reduced or abolished. An antigen of the same size was recognized by serum and CSF from a horse with clinical EPM but not by European horse sera. (Figure 19 A).

Clones from a cDNA library constructed from the mRNA of *S. neurona* merozoites cultured in vitro were randomly sequenced. Partial sequence of one clone containing a 687 bp insert when translated into an amino acid sequence had 32% identity to the major surface antigen of *S. muris* (SmMSA1). This partial clone was used as a probe to obtain a full-length copy of the coding region of the gene. Screening the library by hybridization with this probe demonstrated that ~10% of clones screened bound this probe. Analysis of seven positive clones revealed that many of the cDNA inserts were partial clones of similar length (~0.83 kb), and they shared a common restriction map (data not shown). One larger clone had an extended 5'-untranslated region with a total insert size of 1232 base pairs. The translated peptide is shown (Figure 20). The sequence of this longer clone contains a 5' noncoding region of 72 bp, a single long open reading frame of 828 bp, and a 333 bp 3' nontranslated region. When the probe was hybridized to a blot of total RNA isolated from *S. neurona*, one extremely strong band was detected at 1.5kb (Figure 21A). It thus appears that the cDNA clone we have sequenced contains most of the major mRNA transcript. This probe also hybridized to *S. neurona* DNA fragments but not to bovine host cell DNA. A blot of EcoR1-cleaved DNA hybridized with this probe yields a single band at approximately 1.2 kb (Figure 21B).

As shown in Figure 20, the ATG codon at the beginning of the reading frame (bold italic) is preceded by two in-frame termination codons at positions –39 and –66 in the 5' upstream region. The 828 bp reading frame codes for a polypeptide of 276 amino acids with a calculated molecular mass of 28,328 daltons and an isoelectric point (pI) of 7.48 as predicted by ProSite. The predicted protein is a type 1a membrane protein with a putative signal peptide, one transmembrane spanning region at its C terminus. It has a potential cleavage site at residue 248. The sequence has 12 cystine residues 10 of which are positionally conserved when compared with SmMSA-1 and 9 are conserved when the comparison is extended to NcGRA-1 (data not shown).

Over-expression of SnMSA-1 in *E. coli* using pET 14b produced a recombinant protein of 29 kDa, pI 7.3, with an N-terminal extension containing 6 His residues and a thrombin cleavage site. Purification of the recombinant protein was achieved using a His-tag column (Figure 24). The His-tag was confirmed by N-terminal sequencing and was recognized on immunoblots by His-tag monoclonal antibody. Carbamylated protein standards were used to determine the pI of rSnMSA-1 focused on IPG strips at pI 7.3 (Figure 25).Thrombin cleavage of the recombinant protein to release the His-tag yielded rSnMSA-1 that was recognized on immunoblots by mAbs 1631, 2A7, polyclonal monospecific mouse anti-rSnMSA1, equine serum and CSF, but not serum from European horses or monoclonal isotype controls (Figure 22). Cleavage of the His-tag from the recombinant protein decreased the apparent molecular weight slightly as detected by SDS-PAGE analysis.

Monospecific anti-rSnMSA-1antisera bound native *S. neurona* surface antigens by IFA, post embedding immunogold labeling and ELISA confirming the identity of this clone as a surface antigen of this parasite (data not shown).

Discussion

SnMSA1 is an immunodominant, 29 kDa, merozoite stage antigen recognized on protein blots when probed with intrathecal antibodies from horses naturally infected with *S. neurona* and exhibiting signs of EPM. Monoclonal antibodies 1631 and 2A7 raised against whole merozoites and polyclonal antisera raised against rSnMSA1 both bind a protein of 29 kDa on blots of native *S. neurona* antigens and the purified rSnMSA-1 separated by SDS-PAGE under non-reducing conditions. Monoclonal antibodies that bound rSnMSA-1 also bound to the surface of *S. neurona* merozoites by IFA and post embedding immunogold labeling. These data, when taken together, indicate that the gene SnMSA1 codes for a 29 kDa surface antigen of *S. neurona*.

The cultured merozoites of *S. neurona* have provided material for the immunoblot testing of horses to demonstrate the presence of antibodies that are present in infected horses.[12] Antigens that are considered diagnostic of exposure and consistent with disease are those that range at approximately 29-30 kDa and 19 kDa.[22,51] Many horses demonstrate the presence of antibodies to a 29 kDa antigen of *S. neurona* indicating that it is an immunodominant antigen.[50] The gene encoding SnMSA-1 is found on a 1.2 kb EcoR1 fragment of genomic DNA but the copy number and genomic DNA sequence are yet to be determined. There is a high level of expression of SnMSA-1 so as demonstrated on our RNA blot and our library screening results. This is further supported with data

provided from the recent appearance of 1700 sequence tags of *S. neurona* in the dbEST at NCBI. When sequence from SnMSA-1 is compared to the dbEST database using the BLAST algorithm the result identifies 101 sequence tags of SnMSA-1.

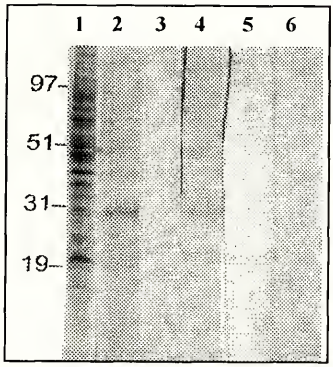
We characterized the thrombin cleaved recombinant protein as having a molecular mass of approximately 28 kDa and an isoelectric point (pI) of 7.3. The predicted structure is that of a type 1a membrane protein with a signal peptide and one transmembrane spanning region at its C terminus. It has a potential membrane cleavage site at residue 248. Antibodies in serum and CSF from horses with clinical EPM bind to rSnMSA-1 on protein blots but serum antibodies from European horses never exposed to *S. neurona* do not bind. We found that the monoclonal antibodies bound p29 and rSnMSA-1 best under non-reducing conditions indicating that conformation of the protein may be important for antibody recognition.

This gene may provide a tool with which to study the pathogenesis of disease in the horse. It may be possible to obtain strong evidence of exposure to *S. neurona* by demonstrating the presence of intrathecal antibodies to rSnMSA-1 in horses tested. Studies need to be done to demonstrate the relationship between horses with histopathologically confirmed cases of EPM and antibodies in serum and CSF to rSnMSA-1.[82,83] Further research is needed to establish whether SnMSA-1 is cross-reactive with antisera from horses infected with other apicomplexan parasites. Additionally, it will be important to determine which stages of *S. neurona* express this antigen and how this antigen differs among isolates. Monoclonal antibodies 1631 and 2A7 may be used to screen isolates for expression of SnMSA-1. Also, mAb 1631 can be used in a practical assay using rSnMSA-1 to detect an antibody that specifically binds

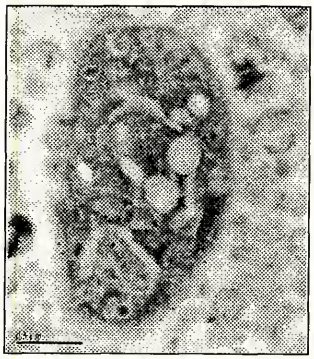
SnMSA-1 in equine serum and CSF. We are currently exploring the use mAb1631 in affinity chromatography to detect minute amounts of antigen in active *S. neurona* encephalitis, this assay could be important to evaluate response to drug therapy in the horse. Other studies using mAb 1631 in immunohistochemistry assays to test the presence of SnMSA-1 in wild life tissues and organisms isolated from these tissues identified as *S. neurona* could be beneficial in our understanding of the biology of this parasite.

Probes prepared from the gene encoding SnMSA-1 may definitively identify *S. neurona* sporocysts or sarcocysts. Currently accepted criteria used to identify *S. neurona* sporocysts are based on amplification of several genomic DNA components followed by restriction digest and sequencing of the amplicons. The identities of sporocysts are only complete, however, when infection studies confirm the known biology of *S. neurona*. We have used PCR data to confirm the presence of SnMSA-1 in four isolates of *S. neurona* (data not shown). The same primers did not amplify a product when genomic DNA from *T. gondii* or *Neospora hughesi* was used. Naturally occurring allelic variants of the SnMSA1 gene may complicate attempts to use this gene as a definitive marker for *S. neurona*, thus additional isolates need to be examined.

A.



B



C.

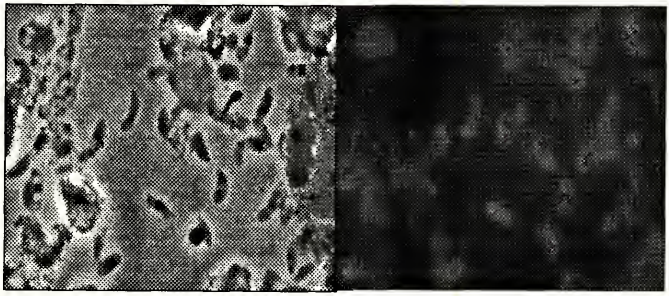


Figure 19. Samples of SDS solubilized *S. neurona* merozoites were analyzed by 4-12% SDS-PAGE under nonreducing conditions, Panel A, Lane 1,and stained with colloidal blue. Antigens were transferred to PVDF membranes and probed with polyclonal antibody, lane2, pre-immune sera, lane3, monoclonal antibody 2A7, lane4, and an isotype control, lane 5. Host cell antigens were run as a control in lane 6. Monoclonal antibody, 2A7, was detected on the surface of parasites with post embedding immunogold labeling, Panel B, and by IFA, Panel C.

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GGGAGGTAAGTGTTGGCGGTAATGCTGCATCATTAGGGTCAGACACGCTGTCCATCTGTC
      *               *
ATTCTCGCCAGA

atgacgagggcggtgctgctgacgtttctgacactctgctccgccagagtgtcccttgtg
 M  T  R  A  V  L  L  T  F  L  T  L  C  S  A  R  V  S  L  V
agggccggagcgccgcctcaagcaacgtgcgccaatggcgaaacgactgttactaagctc
 R  A  G  A  P  P  Q  A  T  C  A  N  G  E  T  T  V  T  K  L
ggcagctctggcgcactacgaatccactgcccaaataatttttcgactcgcgccccgggct
 G  S  S  G  A  L  R  I  H  C  P  N  N  F  R  L  A  P  R  A
gggaatgacgccggtcagatgcaggtctatgcaactgcggttgctgagaatcctgtaaac
 G  N  D  A  G  Q  M  Q  V  Y  A  T  A  V  A  E  N  P  V  N
atacgagacgtcctgcccggcgcatcttacctctctgtacagaacgtcccgaccctcacc
 I  R  D  V  L  P  G  A  S  Y  L  S  V  Q  N  V  P  T  L  T
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 V  P  Q  L  P  A  K  A  T  S  V  F  F  H  C  Q  Q  Q  P  D
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 S  K  E  Q  A  L  P  S  G  A  A  L  A  P  K  D  G  G  L  H
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ggtgtgcaggcagaggcggcccaacggtgtgaggttcgcatctccgtcgcagcgaaccca
 G  V  Q  A  E  A  A  Q  R  C  E  V  R  I  S  V  A  A  N  P
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 D  G  S  V  P  G  A  N  G  A  A  S  L  G  A  A  A  R  S  A
tctgcgttagggttggctctcgttgcaggcgctttcttgcacttttgctaa
 S  A  L  G  L  A  L  V  A  G  A  F  L  H  F  C  *

TCCTGCCGTGTAGCGTCTCTGGTGGCCCGCCCCACAGATCCTGGTTATTCCCACAGCTGC
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TTCCAAAACGGGAAAATCCGAAGATGCAAAATTCATCCGGTGCAGCGTCCCATGTGTTCA
GTTACGACTGGACGAGTGTAGTCACATGGTTTTACATCCATTCGCAGTGCAGAGGCGTGG
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Figure 20. Sequence of SnMSA-1.

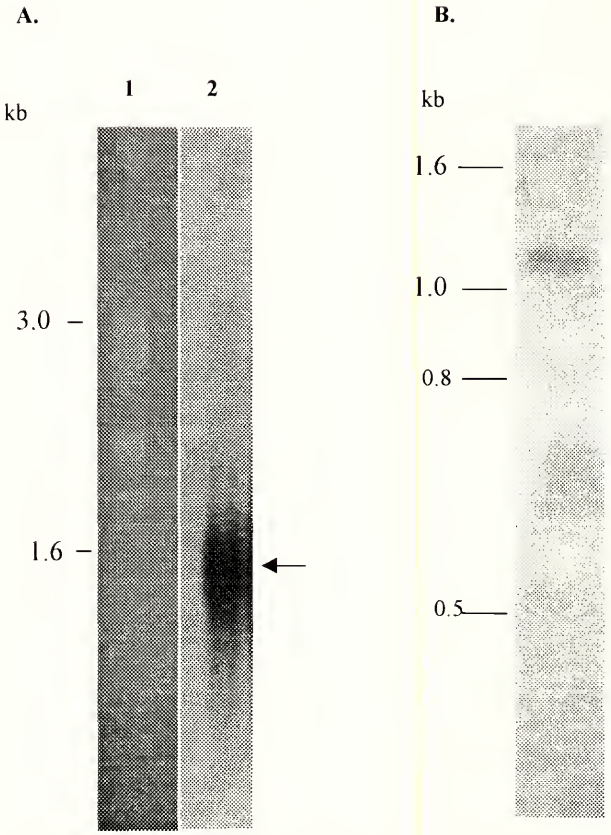

Figure 21. SnMSA-1 gene present in mRNA and genomic DNA. A probe prepared from the SnMSA-1 open reading frame strongly hybridized to *S. neurona* RNA and genomic DNA. Panel A, lane 1 total RNA separated by agarose gel electrophoresis stained with ethidium bromide. Lane 2, SnMSA-1 gene probe hybridized to a Northern blot of total RNA. Panel B, blot of genomic DNA cleaved with EcoR1. When the DNA was cleaved by the enzyme EcoR1 which has no recognition sites within the cDNA insert, a single band was observed.

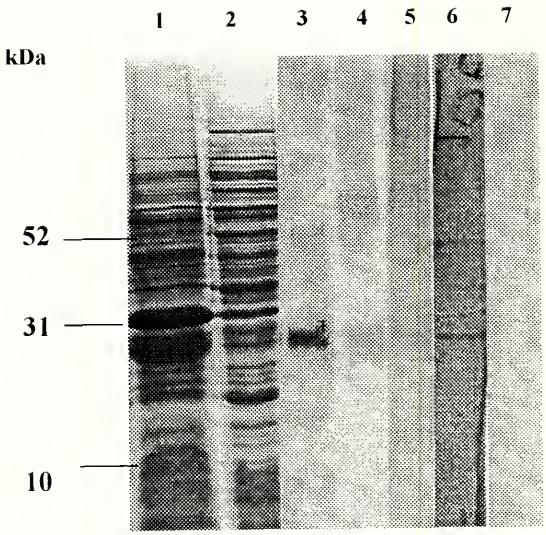

Figure 22. SDS-PAGE analysis of cell lysates of *E. coli* expressing rSnMSA-1, stained with coomassie blue, lane 1 after induction with IPTG; lane 2 is the uninduced clone. Purified rSnMSA-1 was used in Western blot analyses in lanes 3-7. Blots were probed with His-tag monoclonal antibody, lane 3; 2A7 monoclonal antibody, lane4; polyclonal monospecific anti-rSNMSA-1, lane 5; equine CSF, lane 6; serum from European horses, lane 7.

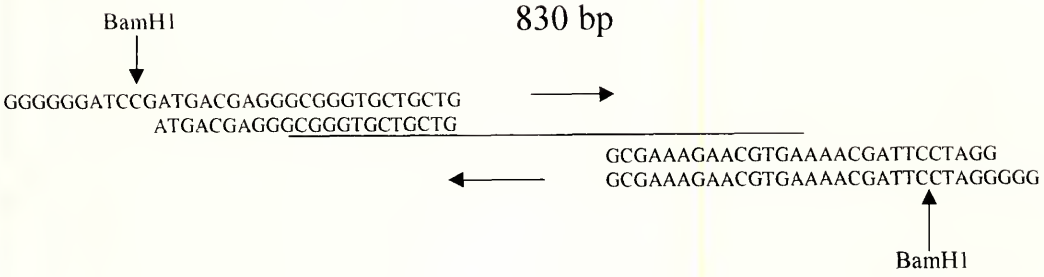

Figure 23. Strategy used to sub-clone the open reading frame of SnMSA-1 into an expression vector, pET 14b.

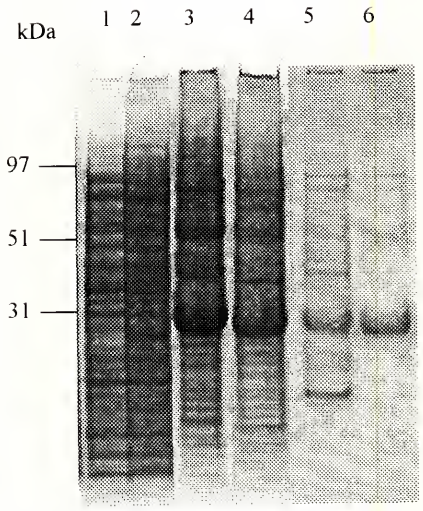

Figure 24. SDS-PAGE analysis of *E. coli* expressing SnMSA-1 as a 29 kDa recombinant protein. Lane 1 uninduced culture; lane 2, induced with 0.4mM IPTG; lane 3, insoluble inclusion bodies; lane 4, sucrose purified inclusion bodies; lane 5, inclusion bodies after solubilization in 8M urea; and lane 6, the recombinant protein captured on a His-tag column.

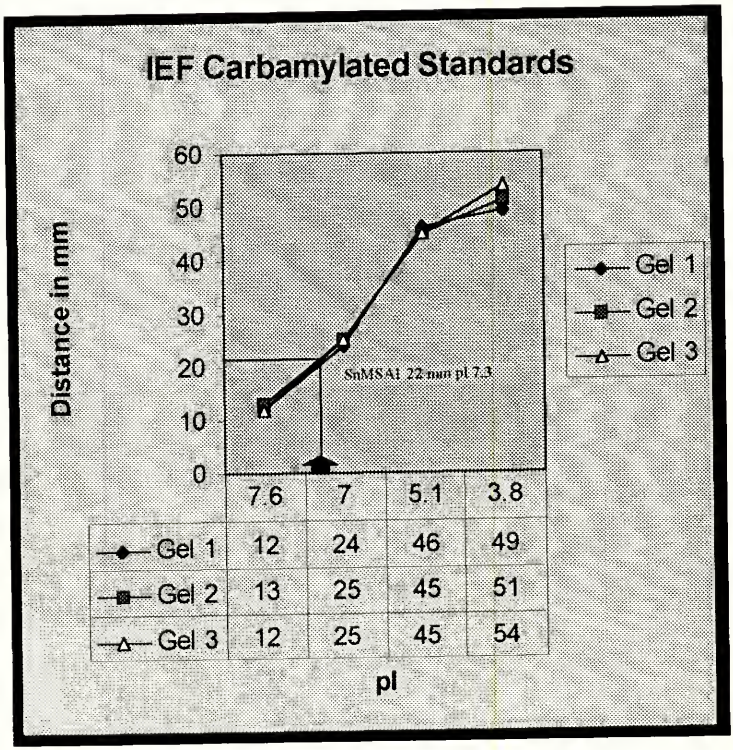

Figure 25. Carbamylated protein standards were used to determine the pI of rSnMSA-1 focused on IPG strips.

CHAPTER 5
CONCLUSIONS

The disease, equine protozoal myeloencephalitis causes neuromuscular disease in horses by infection with *Sarcocystis neurona*. *Sarcocystis neurona* is closely related to *Toxoplasma gondii*, and opinion is still developing with respect to *S. neurona's* phylogenetical status as new information is obtained.[84] Two stages have been identified, the asexually proliferating schizont in the brain and spinal tissue of horses and the sexually produced sporocyst stage, (containing sporozoites) found in the small intestine of the opossum. Much progress has been made since the discovery of *S. neurona* as the etiological agent of EPM but poor diagnostics, ineffective treatment protocols and no means to prevent infection are a result of missing information about the basic biology of this organism.

In order to answer many questions regarding the basic biology of *S. neurona* and the pathogenesis of disease in the horse molecular tools need to be developed. Up to this point the major technological advances have been: 1) culturing the organism *in vitro*; 2) finding a susceptible laboratory species for *in vivo* inoculations; 3) developing an immunoblot assay to detect the immune response of the horse to the parasite; and 4) developing PCR-based tests to discriminate *S. neurona* from other closely related species found in the definitive host, the opossum (*Didelphis virginianum*).

Because the antigens of *S. neurona* are not characterized, I chose to identify specific antigenic components of *S. neurona* and to molecularly characterize these antigens of the infectious stage of the parasite. This information will be used to elucidate the immunological and pathogenic events during infection and help prepare immunotherapeutic tools to prevent disease.

Novel techniques were used to propagate *S. neurona* merozoites and purify them away from host cells. The novel use of calcium ionophore stimulated the release of parasites facilitating the purification of one stage of *S. neurona*. Not all parasites were released and cultures could be selected for slower growing forms that were refractory to calcium ionophore. Though our intent was to prepare increased numbers of free parasites, this may be a way to examine a second phenotype overlooked in culture. It remains unknown how calcium ionophore stimulates the release of *S. neurona* merozoites from host cells. We did not find parasitophorous vacuoles when parasites were examined by high resolution TEM (data not shown), however we only examined parasites growing in one cell line. It is possible we did not create the environment needed to observe parasitophorous vacuoles, yet created an environment that allowed the use of calcium ionophore for purification.

Data showing the heterogeneity of cultured merozoites, demonstrating the different stages of development in different host cell types, was important for purification and may have application to the pathogenesis of disease in the horse. We found culture conditions could change phenotype, but we did not find a change in the antigens detected with the antibodies that we used. We saw an increase in the number of micronemes with ionophore treatment and expect that when a microneme specific antibody is developed

these phenomena can be followed. We were able to grow *S. neurona*, UCD1, in a cell line that dramatically decreased p29 as detected by IFA (S2/O cells, data not shown). Immunization of a mouse with live infected S2/O cells yielded a serum ELISA titer of greater than 1:32,000 to whole parasites grown in BT cells, however the polyclonal mouse anti-*S. neurona* did not bind the parasites by post-embedding immunogold labeling at a 1:10,000 dilution. Although much work needs to be done to establish that our observations were due to a change in the stage of the merozoites cultured in S2/O cells or perhaps a stage specific alteration of gene expression, these findings could lead to insight about the developmental regulation of gene expression in *S. neurona*.

It is possible that the immune response of the horse prevents cyst formation by retarding the development of the merozoites. In this case, the selection of a minute number of merozoites that maintain virulence could occur. This is the case in *T. gondii* where researchers have demonstrated virulence is due to one extra copy of a 27 bp repeat in the promotor region upstream of the SAG-1 start codon.[86] The virulent strains of *T. gondii* have five copies of the repeated sequence whereas avirulent strains have only four; the extra repeat sequence was directly related to the increased expression of SAG-1, a protein important in host cell invasion.[87-89] It is possible the virulence of *S. neurona* parallels that of SAG-1 in *Toxoplasma*. Perhaps, if this were the case the selective pressure of the equine immune system would be required to select the virulent strain of *S. neurona*. Alternatively, if one could allow the virulent strains already isolated from horses to proceed to the sporocyst stage, Kochs postulates could finally be fulfilled. Unfortunately, current technology is lacking to complete the life cycle with isolates of *S. neurona* recovered from equine neurological tissue and propagated in *vitro*. To show that

S. neurona has virulence factors associated with the promotor region of the gene, it will be necessary to compare the 5' upstream region of the virulent *S. neurona* (those isolates cultured from the spinal cord of horses) and the same region of DNA from *S. neurona* merozoites cultured from sporozoites that have not infected horses (when overwhelming numbers were used). More studies are needed to determine the stages of *S. neurona* observed in cultures, the antigens expressed by these stages, and the relevance of virulence factors to the antigens to which horses with clinical EPM respond.

Using mature cultures of UCD 1, a *S. neurona* isolate cultured from the spinal cord of a California horse with clinical EPM, polyclonal antibodies were produced in a rabbit. The rabbit anti-*S. neurona* antibodies were used to identify antigens of *S. neurona* separated by SDS-PAGE in both one and two dimensions. Additionally, the rabbit anti-*S. neurona* antisera was used to immunoprecipitate proteins of UCD 1 in an effort to concentrate the proteins for immunoblotting. Differences were seen in immunoblot results when antibodies from animals immunized with killed merozoites were compared with natural infections. This could be due to differences in the antibody populations, those of a rabbit versus those of a horse, that were used or to binding properties of serum antibodies versus thecal antibody produced in the horse. The serum and CSF of diseased animals was used to identify antigens important in natural infections allowing us to consider the 19 kDa antigen in our experiments. Certainly, the 19 kDa antigen immunoprecipitated by equine CSF is important in infection if serum neutralization work done by Liang and work done by both the groups of Marsh and Rossano are correct.[22,50-51] Another consideration is the form of the 19 kDa protein, it may be expressed at a low level until host cell invasion. In this case using killed antigens did not produce the tools needed to investigate p19. It is

possible immunization using live organisms in rabbits, horses, or mice may be needed to make antisera. If mice are used for the investigations, the use of S2/O cells has an advantage in eliminating the mouse response to cultured host cells. If the eventual use of the immunized animal is for the production of monoclonal antibodies, screening the hybridomas should be simplified with the elimination of clones against host cells.

Monoclonal antibodies were essential in my effort to relate the sub-cellular location of native antigens to SDS-PAGE separated proteins as well as to clones expressing the corresponding recombinant protein. We produced 4F1MC, antibodies to a species-specific surface protein. This surface protein was not glycolipid anchored nor soluble in 1% Triton X-100. The production of a similar, if not identical, monoclonal antibody by another group allowed us to continue our work on p29 when we were unable to clone 4F1. The second monoclonal bound p29 under non-reducing conditions and confirmed that this antigen is dominant in an immune response when killed organisms are used for immunization. The production of recombinant protein expressing the epitope that reacted to 4F1 and the second monoclonal allowed us to recover cloned 4F1 monoclonal antibody, Mab 1631.

Our approach identified a 29 kDa major surface antigen of *S. neurona* that was species specific. Most recently, our observations and conclusions were supported by others.[51] In order to locate and characterize the major surface antigen expressed by *S. neurona* merozoites, we made a cDNA expression library. Calcium ionophore release of parasites from host cells enabled the production of a cDNA expression library with minimal host cell contamination. The expression library was screened with both polyclonal rabbit anti-*S. neurona* and mass culture supernate from hybridoma cells

produced from mice immunized with UCD 1. Approximately ten percent of clones were identified as binding antibody when plaques were lifted and resulting blots were probed with 4F1MC, indicating that the polyadenylated transcript is abundant in cultured merozoites.

A gene fragment with sequence similarity to the major surface antigen of *S. muris* was found during the library analysis. The plasmid containing the 5' region of SnMSA-1 was labeled and used as a probe to select a full-length copy of the gene. Interestingly, the labeled clone hybridized with approximately ten percent of the clones screened, supporting the earlier observation that this is a highly expressed gene. Sequence data from seven of the reacting clones contained partial fragments of SnMSA-1. Additionally, recent data from 1750 clones derived from a *S. neurona* sequence tag project posted on NCBI web site contained 131 clones identical to partial sequence of SnMSA-1, this represents 12% of the published sequences from this library after correction for duplicate sequences are subtracted. It is possible that using the calcium ionophore to prepare parasites for construction of our library skewed the representation of the genes, however one would expect an overabundance of microneme transcripts. This was not detected. Additionally, mice, rabbits, and clinical sera support our data because the 29 kDa antigen was immunodominant when these sera were used in immunoblots with native antigen.

The sequence data from the full length gene was used to design PCR primers. The primers were then used with the second strand synthesis reaction (used in library construction) as template to produce an amplicon containing the open reading frame of SnMSA-1 with flanking BamH1 restriction sites. This approach allowed the gene to be sub-cloned into the BamH1 site of the expression vector pET 14b and the recombinant

protein produced. The recombinant protein, rSnMSa-1, was shown to have an expected mass of approximately 29 kDa. Monoclonal antibody that binds the surface of *S. neurona* cultured merozoites by IFA and post embedding immunogold labeling electron microscopy was also shown to bind a 29 kDa antigen of SDS-PAGE separated native *S. neurona* proteins under non-reducing conditions. Monoclonal antibody to an epitope of a 29 kDa protein from the cultured merozoites of *S. neurona*, p29, was used to verify the presence of the epitope on the recombinant protein. Monoclonal antibody did not detect the epitope by IFA when reacted with *S. falcatula*, *Toxoplasma gondii*, and *Neospora hughesi* merozoites. Serum and CSF from horses with EPM, but not European horses, recognized rSnMSA-1. The recombinant protein was purified and used to produce a monospecific polyclonal antibody in a mouse. The mouse anti-rSnMSA-1 was used to characterize the native antigen, p29, of cultured *S. neurona* merozoites as a surface protein. Taken together, these data support the conclusion that SnMSA-1 is a major surface antigen of *S. neurona*. Horses that have been exposed to *S. neurona* have a measurable immune response to this antigen.

A plasmid isolated from the transfected clone producing the recombinant protein was labeled and used as a probe to verify the gene in the mRNA of *S. neurona* UCD 1 by Northern analysis. It was also used to demonstrate the presence of SnMSA-1 as a low copy number gene on a Southern blot of EcoRI digested DNA prepared from cultured *S. neurona* merozoites. Further, primers used to generate the full length open reading frame of SnMSA-1 also produced an approximately 850 bp amplicon when genomic DNA prepared from the cultured merozoites of three isolates of *S. neurona* was used as a

template. Additional analysis of sequence data is required to verify that there are no allelic differences among different isolates.

No amplicon was detected when genomic DNA prepared from the cultured merozoites of *S. falcatula*, *Toxoplasma gondii*, and *Neospora hughesi* was used as a template, although suggestive, these data are incomplete. It will be necessary clone and compare the corresponding gene from these other apicomplexan parasites before it can be determined how similar it is to its SnMSA-1 counterpart. The ELISA data we obtained using monoclonal antibodies to rSnMSA-1 against cultured merozoites of *S. falcatula*, *Toxoplasma gondii*, and *Neospora hughesi* supports the negative PCR data to indicate that SnMSA-1 is sufficiently different so as to be useful in discriminating between *S. neurona* and these species. Tissue sarcocysts from the armadillo (*Dasypus novemcinctus*) were identified as *S. neurona*.[89] PCR reactions using cDNA prepared from these sarcocysts identified as *S. neurona* or DNA prepared from sporocysts derived from the sarcocysts also produced an amplicon of 830 bp. These data verify that SnMSA-1 is present in multiple isolates of *S. neurona*, all known stages as identified by genomic DNA, but not other apicomplexan parasites known to infect the CNS of horses. Additional work needs to be completed to establish that SnMSA-1 is present in all isolates of *S. neurona* from horses with EPM. Further work needs to be done elucidating the expression of this gene in different stages of *S. neurona*. Additional work includes the examination of serum and CSF from natural infections caused by *Neospora* versus rSnMSA-1. *Neospora* and *Toxoplasma* share homology in their major surface antigens and one can not yet rule out the possibility that *S. neurona* does also.[90-95] In summary, the work presented in this dissertation characterizes a surface antigen of *S. neurona* and supports the use of

rSnMSA-1 and SnMSA-1 in the diagnosis of *S. neurona* encephalomyelitis in horses. The immediate practical application of the recombinant protein SnMSA-1 was briefly examined. The recombinant protein was used as the antigen in an ELISA and immunoblot to measure antibody in body fluids of clinically normal, sporocyst challenged, and infected horses. Further, an ELISA was used to quantitate the amount of antigen in a sample prepared for use in immunoblots. The methods used in this study will facilitate further investigation of the genes of *S. neurona*. It is hoped rSnMSA-1 will be useful to standardize the amount of parasite antigen used on immunoblots for the detection of *S. neurona* exposure. Our data indicates the environment in which proteins are separated by SDS-PAGE can influence the interpretation of results of equine exposure to *S. neurona* and suggest rSnMSA-1 detection of antibody by ELISA may improve the diagnosis of EPM in horses. The accumulation of genetic information on this parasite will provide the basis for specific diagnosis, treatment and prevention of disease in horses and to this end our cDNA library is a valuable contribution.

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BIOGRAPHICAL SKETCH

Siobhan P. Ellison was born December 13, 1952, in England. She obtained her BS and MS at the University of Florida in veterinary microbiology. She worked as a research assistant in biochemistry followed by her return to the University of Florida where she obtained a DVM degree in 1983. She has maintained an ambulatory equine practice in Ocala Florida where she has dedicated her life to the care and welfare of horses. She hopes to continue her contribution to the equine industry by developing tools for the diagnosis and prevention of disease in horses.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

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